AWARD NUMBER: W81XWH-12-1-0191

TITLE: Treatment and Prevention of Breast Cancer Using Multifunctional Inhibitors of Cholesterol Biosynthesis

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REPORT DATE: August 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2015	Final	1Jun2012 - 31May2015

4. TITLE AND SUBTITLE

Treatment and Prevention of Breast Cancer Using Multifunctional Inhibitors of Cholesterol Biosynthesis

5a. CONTRACT NUMBER

W81XWH-12-1-0191

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Missouri, Columbia
Columbia, MO 65211-1230

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Most human breast cancers are hormone responsive, with proliferation of tumor cells dependent on estrogens and progestins. Hormone-responsive tumors respond initially to endocrine therapy, though most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase in the cholesterol biosynthetic pathway, effectively arrested breast cancer cell proliferation. In the 2014-2015 Annual Reports we discussed a number of findings establishing that an OSC inhibitor, RO 48-8071 (RO) possesses potent anti-cancer properties. These results have been published in Breast Cancer Research and Treatment, a highly reputable journal, and are appended in this report. A second report was also published in the journal Oncology and is also appended. During the no-cost extension period we concentrated on finalizing the immunohistochemical studies to determine mechanism of action of RO. We show that therapy involving a combination of RO and an estrogen receptor-beta agonist is an extremely effective means of treating breast cancer. Our studies show that estrogen receptor beta is induced by RO (or in some cases levels are not affected). Importantly, the pro-proliferative estrogen receptor-alpha is destroyed by RO.

15. SUBJECT TERMS

Nothing Listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	55	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

[SF298]
Note: An abstract is required to be provided in Block 14

Most human breast cancers are hormone responsive, with proliferation of tumor cells dependent on estrogens and progestins. Hormone-responsive tumors respond initially to endocrine therapy, though most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase in the cholesterol biosynthetic pathway, effectively arrested breast cancer cell proliferation. In the 2014-2015 Annual Reports we discussed a number of findings establishing that an OSC inhibitor, RO 48-8071 (RO) possesses potent anti-cancer properties. These results have been published in Breast Cancer Research and Treatment, a highly reputable journal, and are appended in this report. A second report was also published in the journal Oncology and is also appended. During the no-cost extension period we concentrated on finalizing the immunohistochemical studies to determine mechanism of action of RO. We show that therapy involving a combination of RO and an estrogen receptor-beta agonist is an extremely effective means of treating breast cancer. Our studies show that estrogen receptor beta is induced by RO (or in some cases levels are not affected). Importantly, the pro-proliferative estrogen receptor-alpha is destroyed by RO. These studies show for the first time that inhibition of cholesterol biosynthesis using OSC inhibitors is a novel and potent means by which to destroy human breast cancer cells, and, furthermore, that a combination of RO with agonists of estrogen receptor beta is a viable treatment option that should be considered for breast cancer patients who display estrogen receptor in their biopsies or exhibit properties of triple-negative breast cancer. Finally, we have also established that RO controls not only estrogen receptor activity, but also activity of the androgen receptor. These results are attached and are of particular importance as the androgen receptor is regarded as a target which might be used to combat triple negative breast cancers, for which there are currently few therapeutic options available.

Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The purpose of this research was to investigate whether RO, an inhibitor of cholesterol synthesis, is also an effective therapeutic drug which might be used to control the progression of breast cancer. The effects of RO on a number of different breast cancer cell lines was examined, as well as its in vivo effect against breast cancer cells grown in xenograft models. Protocols in which the inhibitor was given alone or in combination with other compounds have been employed. Initial results were reported previously. Some of these results have been published and others are in preparation for publication. Two of the manuscripts are appended. During the past 12 no-cost extension months, intensive studies to determine the effectiveness of combination therapy using RO and estrogen receptor beta agonists have been performed. These studies were heavily dependent upon immunohistochemical analysis, which enabled us to determine mechanisms of action of RO. The results are reported herein and manuscripts are under preparation. In addition, efforts have been made to determine whether RO influences the transcriptional activities of estrogen and androgen receptors. These latter studies form the basis of one of the attached manuscripts.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Breast cancer, cholesterol inhibitors, therapeutics, cell viability, apoptosis, estrogen receptor beta, combination therapy, transcriptional activity, androgen receptor.

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Progress related to Task 1. ***Characterize the impact of RO on estrogen signaling in breast cancer cells. (estrogen receptor alpha=ERα; estrogen receptor beta=ERβ)***

Goals:

a. Determine the effect of RO therapy on cell viability using a number of different breast cancer cells, and normal mammary cells.

b. Determine the level of ERα and ERβ expression in treated cells by Western blot analysis.

c. Determine the effect of RO treatment on estrogen-dependent proliferation of breast cancer cells.
d. Determine whether RO treated ERα positive cells lose their capacity to regulate ERα-dependent gene regulation but retain the ability to regulate ERβ specific genes with ERβ-interacting ligands.
e. Determine whether RO influences transcription of ERα and ERβ genes in breast cancer cells.
f. Determine whether RO influences stability of ERα and ERβ protein in breast cancer cells.

Most of the aims were completed and results are published in the two manuscripts attached. (These results are also inserted in part as Figs 1-6). Recently presented abstracts at various national and international meetings are also attached. The studies with respect to point f were inconclusive.

Progress related to Task 2. ***Characterize the in vitro effects of RO mono- or combination therapy on proliferation and apoptosis of breast cancer cells in vitro.***

Goals:
a. Measure apoptosis in breast cancer cells treated with RO alone or RO in combination with ERβ interacting ligands.
b. Determine protein levels of apoptosis related genes (p21, caspase-3, Bcl-2, Bad, Bax) following treatment of cells with RO.
c. Initiate combination therapy, keeping the concentration of one ligand constant while varying that of the other to determine whether there are additive or synergistic effects on apoptosis.
d. Determine mRNA and protein levels of proteins related to apoptosis and angiogenesis, such as p21, caspase-3, Bcl-2, Bad, Bax, and VEGF following treatment of cells with RO in combination with aforementioned compounds.
e. Transfect cells with siRNA to down-regulate ERβ and determine cell viability and response to RO using cell-proliferation assays.
f. Following ERβ and OSC siRNA transfections, test breast cancer cells for lack of sensitivity to RO in order to define a molecular target for mediating RO effects. These experiments will utilize cell viability assays.

Studies described in task 2 have been completed and also reported in the attached manuscript. (also please see Figs 7 and 8).

Progress related to Task 3. ***Characterize the effects of RO mono- or combination therapy on progression and prevention of breast cancer cells in vivo in rodent models.***

Goals:
a. Breast cancer cells in matrigel will be inoculated into nude mice (6-8 week-old, female, nu/nu, sc).

b. Tumors will be allowed to reach 100-200 mm^3 in size, after which animals will be randomly assigned to groups for treatment with RO or vehicle alone. RO treatment (5-25 mg/kg, iv) will be once a day for 10 days.

c. Experiment in b) will be repeated in vivo using a combination of RO and an ERβ specific ligand, as well as RO and a natural compound with an affinity for ERβ, to determine additive or synergistic effects in reducing in vivo tumor progression.

d. Tumor samples will be collected after the first three injections and then again at the end of the experiment in b. and c. for further analysis by immunohistochemistry. Samples will also be saved in liquid nitrogen for Western blot analysis and RNA isolation.

e. Western Blot and RT-PCR will be used to analyze protein levels and RNA expression of ER, PR , p21, Caspase-3 and VEGF.

f. Immunohistochemistry will be used to quantitate blood vessel density and various antigens indicated in e.

We showed in our previous report that RO is effective against breast cancer (manuscript attached). We now have robust data from experiments conducted during the last two years (including the no-cost extension period) showing that combination therapy with RO and an estrogen receptor beta ligand is an efficient way to treat both hormone-dependent and hormone-independent breast cancers without toxicity (Figs 9 and 10). Based on in vitro combination therapy to determine the most effective ERβ interacting ligand, we used liquiritigenin in our subsequent in vivo studies. During the no-cost extension period we also conducted immunohistochemical analysis of tumor tissue to determine mechanistically how such a powerful anti-tumor effect occurs. A combination of RO and liquiritigenin effectively induces tumor cell apoptosis as well as reducing angiogenesis within tumors (Figs 11A and B). These studies are complete and are currently being prepared for publication.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

    - OSC expression is not associated with stage of breast disease
    - RO blocks transcriptional activities of both estrogen and androgen receptors
    - RO blocks estrogen receptor alpha more strongly than estrogen receptor-beta
    - RO competes with estrogen for binding to the estrogen receptor though at much higher concentrations, suggesting an allosteric modification of estrogen receptor
    - RO blocks the production of an estrogen regulated gene (progesterone receptor) in breast cancer cells
    - RO does not regulate estrogen receptor at the transcriptional level
    - RO in combination with estrogen receptor beta interacting agonists is a powerful combination which stops the progression of breast tumors both in vitro and in vivo

- RO in combination with estrogen receptor beta interacting agonists is a powerful inducer of cell death and reduces angiogenesis markers in tumor tissue.

5. CONCLUSION:

Our research, with the support of this grant, shows that inhibitors of cholesterol synthesis that target OSC induce tumor cell apoptosis and can therefore be used to prevent the progression of breast cancer cells. In addition OSC inhibitors have off-target effects; they degrade estrogen receptor alpha, a major pro-proliferative protein in hormone-responsive cells, and induce estrogen receptor beta protein, a major factor which reduces cell proliferation. The results pertaining to combination therapy involving RO and estrogen receptor beta interacting ligands are particularly interesting and important since they potentially support the use of lower levels of toxic chemotherapeutic drugs together with RO to bring about tumor regression. Once the studies proposed in the grant are complete, they will yield information vital to determining the suitability of these drugs for use in humans. We propose that it is important to move forward with human clinical trials which, we believe, could set the stage for the therapeutic use of OSC inhibitors to combat breast cancer. We contend that their use has the potential to save millions of lives worldwide, including the lives of women serving in the US Army who contract breast cancer due to genetic make-up, use of exogenous hormones, or as a result of exposure to carcinogens.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Lay Press:

http://munews.missouri.edu/news-releases/2014/0617-potential-cholesterol-lowering-drug-has-breast-cancer-fighting-capabilities-mu-researcher-finds/

http://news.missouri.edu/2014/a-year-of-discovery/

(2) Peer-Reviewed Scientific Journals:

Liang Y, Besch-Williford C, Aebi JD, Mafuvadze B, Cook MT, Zou X, Hyder SM. (2014) Cholesterol biosynthesis inhibitors as potent novel anti-cancer agents: suppression of hormone-dependent breast cancer by the oxidosqualene cyclase inhibitor RO 48-8071. Breast Cancer Res Treat. 146:51-62.

Mafuvadze B, Liang Y, Hyder SM. (2014) Cholesterol synthesis inhibitor RO 48-8071 suppresses transcriptional activity of human estrogen and androgen receptor. Oncol Rep. 32:1727-1733.

Additional manuscripts are under preparation

(3) Invited Articles:

Hyder, S. M., Mafuvadze, B and Besch-Williford, C. (2013) Novel Anti-Angiogenic Therapies using Naturally-Occurring and Synthetic Drugs to Combat Progestin-Dependent Breast Cancer to be published in Cell and Molecular Biology of Breast Cancer, Humana Press, pp 123-147

(4) Abstracts:

Liang, Y., Zou, X., Besch-Williford, C., Johnnes, A. and Hyder, S. M. (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

Mafuvadze, B., Liang, Y., Hyder, S. M. (2014) Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells. 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo, Chicago, IL. Abstract SAT-0279

Liang, Y., Aebi, J. and Hyder, S.M. (2015) Inhibitors of oxidosqualene cyclase block growth and survival of both hormone-dependent and independent breast cancer cells. Proceedings of the 20th World Congress on Advances in Oncology, Athens, Greece. In press

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

*Liang, Y., Zou, X., Besch-Williford, C., Johannes, A. and Hyder , S. M. (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

*Mafuvadze, B., Liang, Y., Hyder, S. M. (2014) Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells. 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo, Chicago, IL. Abstract SAT-0279

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7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8. REPORTABLE OUTCOMES:

All the results described in Section 3 are reportable. Some of these have been published (attached manuscript), and additional data will soon be published. The results show a substantial advance towards a potentially new therapeutic protocol for breast cancer which could involve the use of specific cholesterol lowering drugs that target oxidosqualene cyclase. These drugs may be administered with or without additional drugs that target estrogen signaling mechanisms. Evidence for this scenario comes from our observation that estrogen receptor beta specific ligands appear to enhance the effects of cholesterol lowering drugs. It is possible that such an approach could also prove useful for preventing breast cancer in the first place.

9. OTHER ACHIEVEMENTS:

Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. REFERENCES:

n/a

11. APPENDICES:

Please see attached

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

COLLABORATIVE AWARDS: n/a

QUAD CHARTS: n/a

Figure 1: Relative OSC mRNA expression in breast cancer at different stages of growth determined using real-time PCR assay
(No significant correlation was obtained)

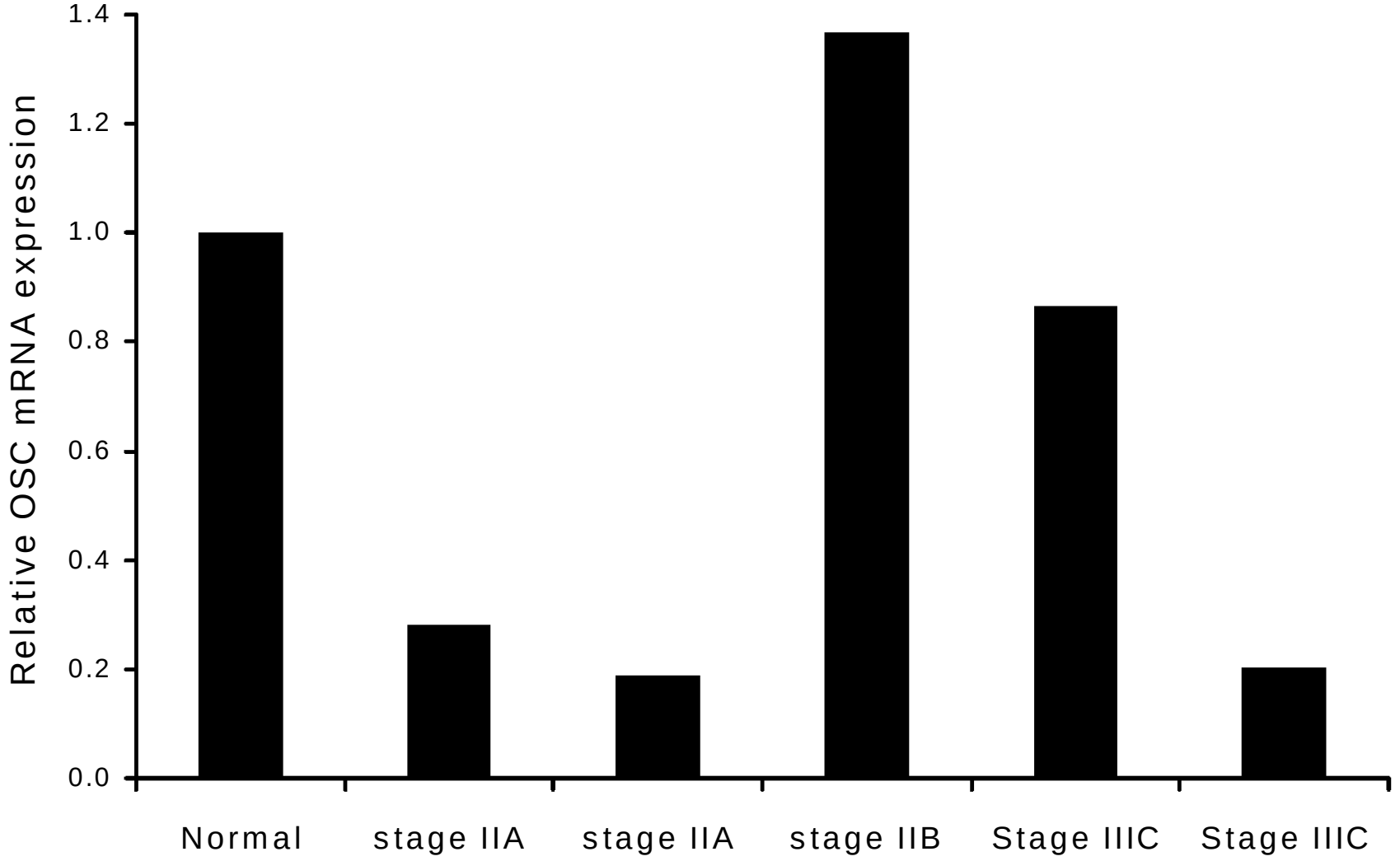


Figure 2: Relative OSC mRNA expression in distinct breast cancer tissues at different stages of growth (Stage I-III).
Results showed no difference in expression between ER/PR positive, ER/PR negative and triple-negative tissues (ER/PR and Her2-neu negative). *triple-negative, **ER/PR negative Her2-neu high; remaining tumors are ER/PR positive

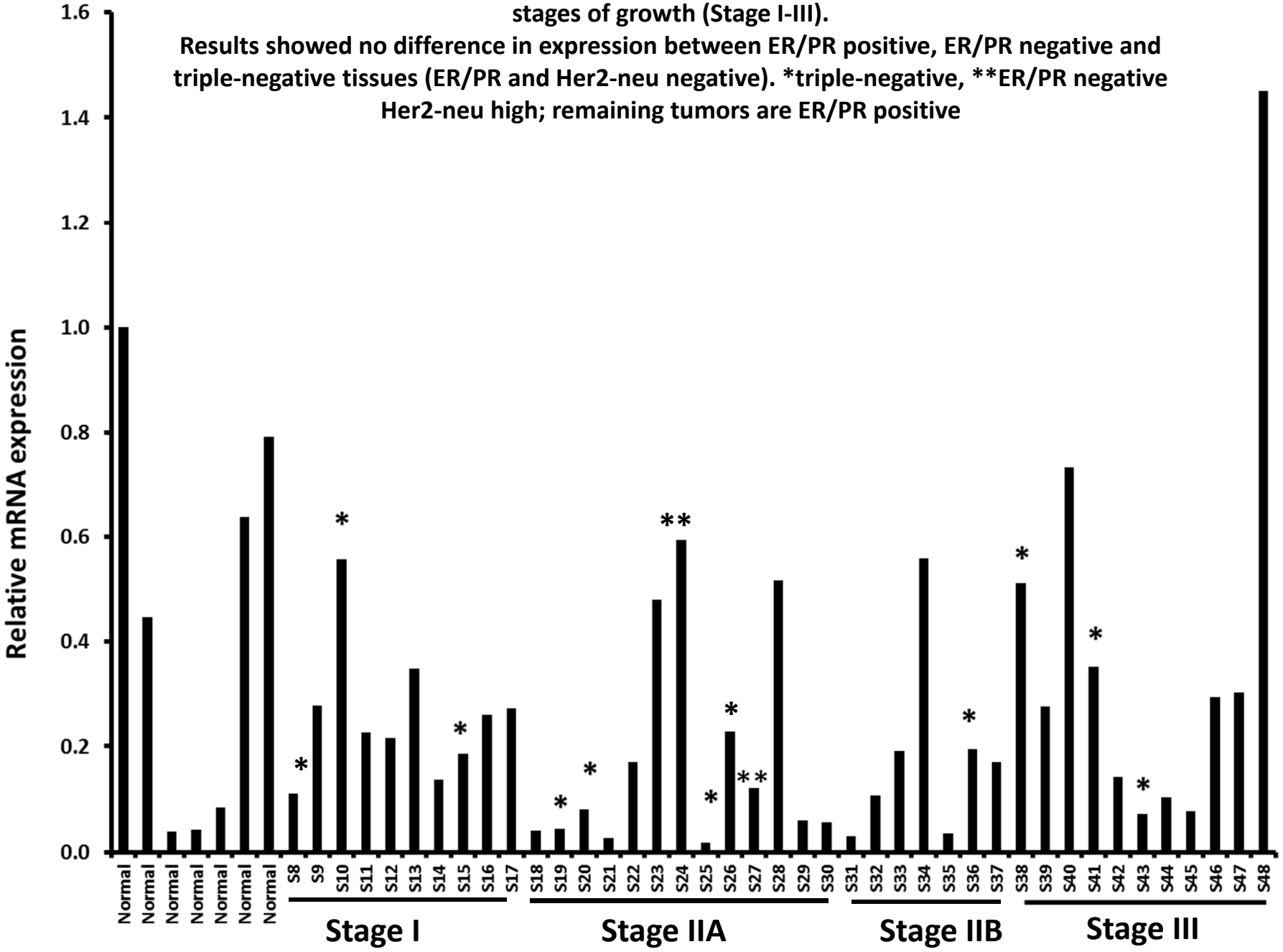


Figure 3A-D: RO significantly inhibits estradiol induced estrogen receptor-mediated transcriptional (luciferase) activity.

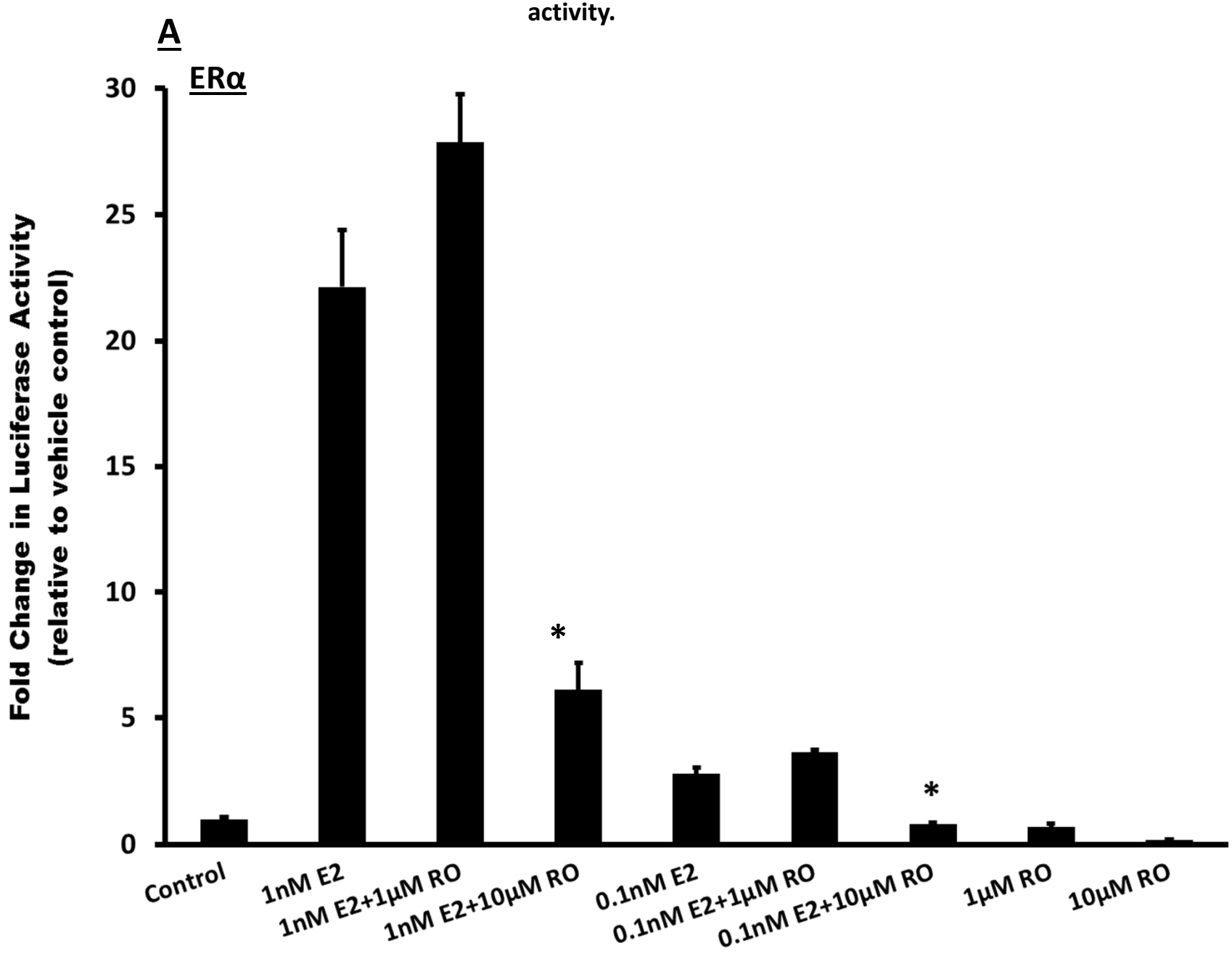


Figure 3B.

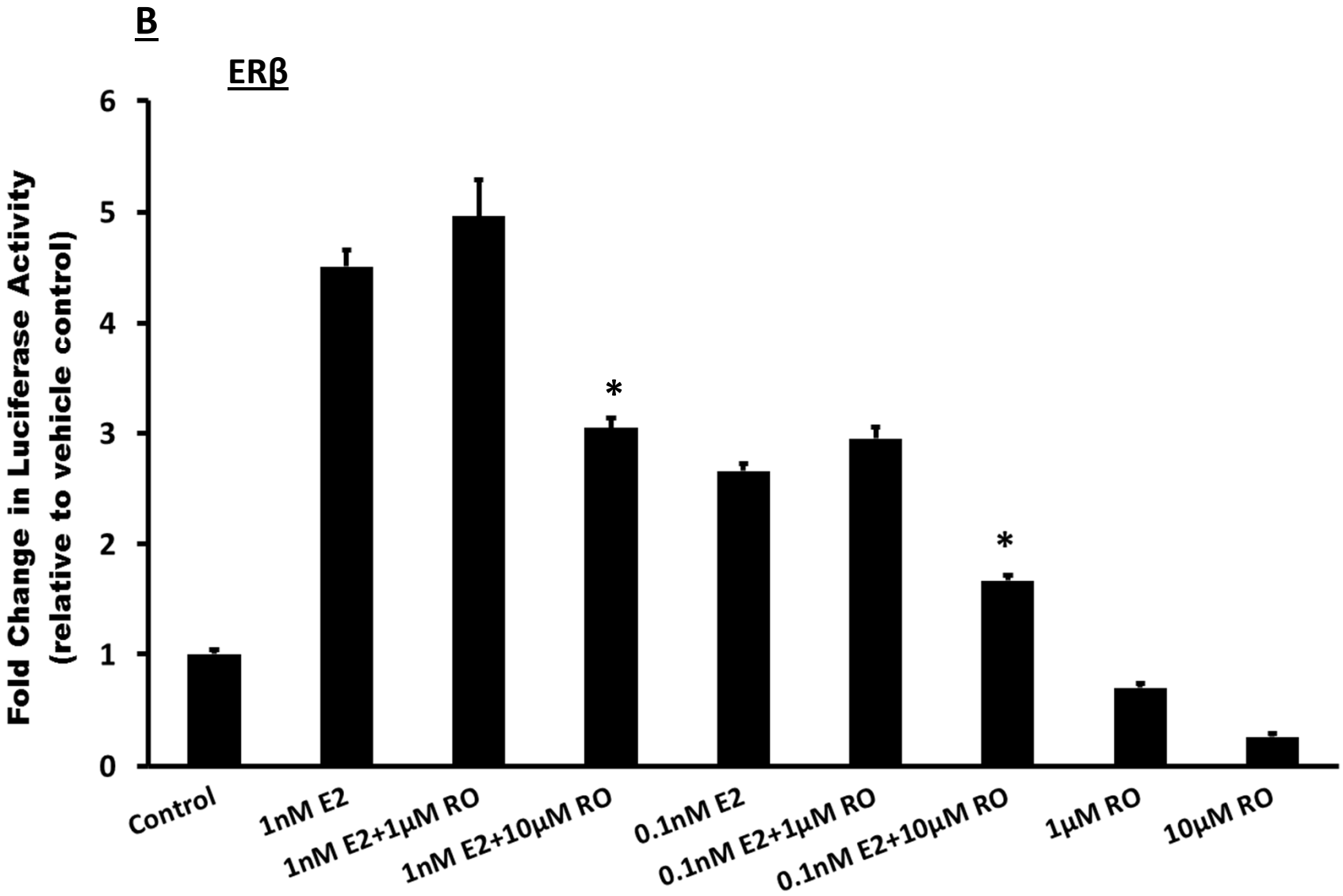

B
ERβ
Fold Change in Luciferase Activity
(relative to vehicle control)
0
1
2
3
4
5
6
*
*
Control
1nM E2
1nM E2+1μM RO
1nM E2+10μM RO
0.1nM E2
0.1nM E2+1μM RO
0.1nM E2+10μM RO
1μM RO
10μM RO

Figure 3C. ICI=ICI 182,780, an anti-estrogen (antagonist)

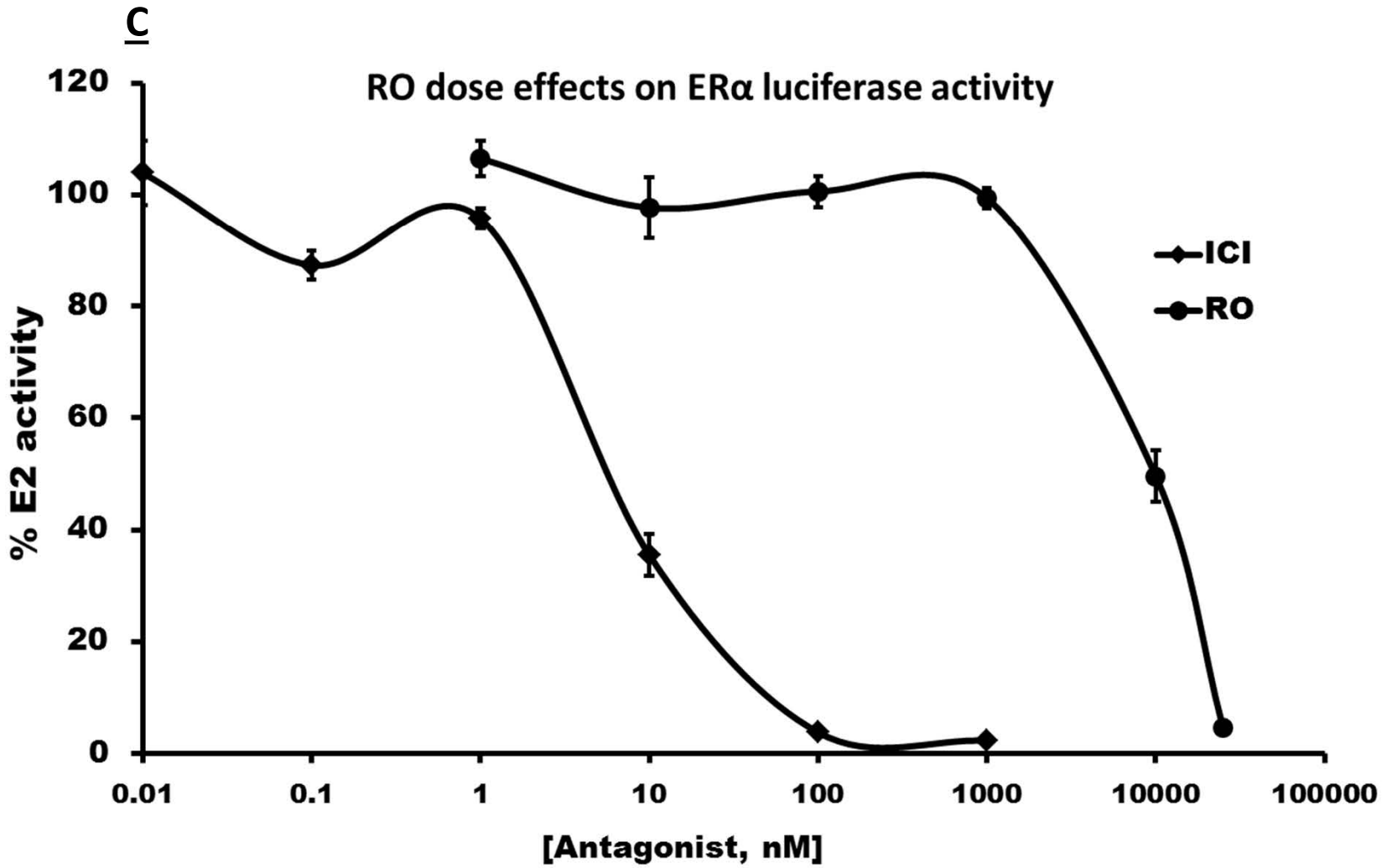
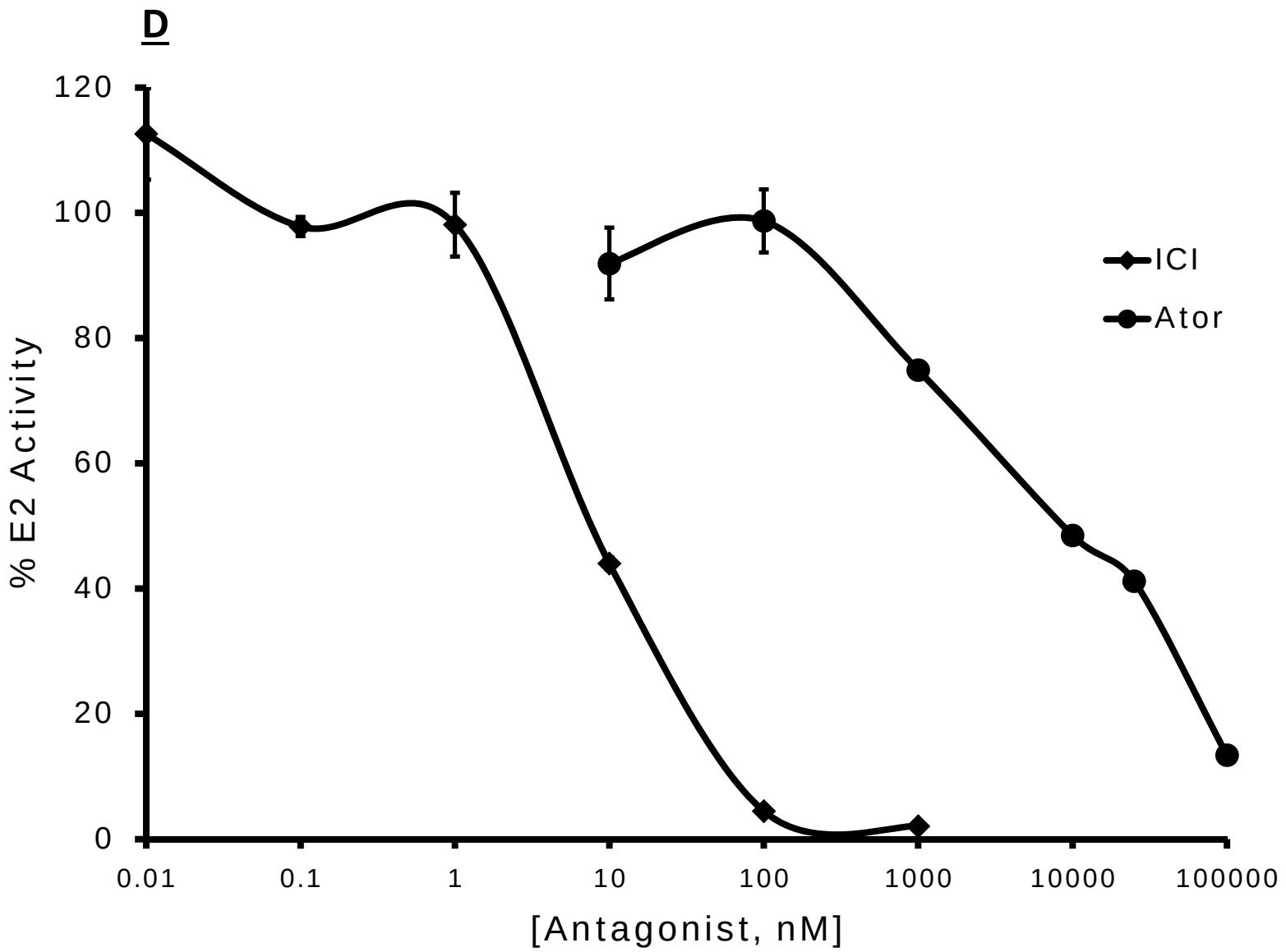
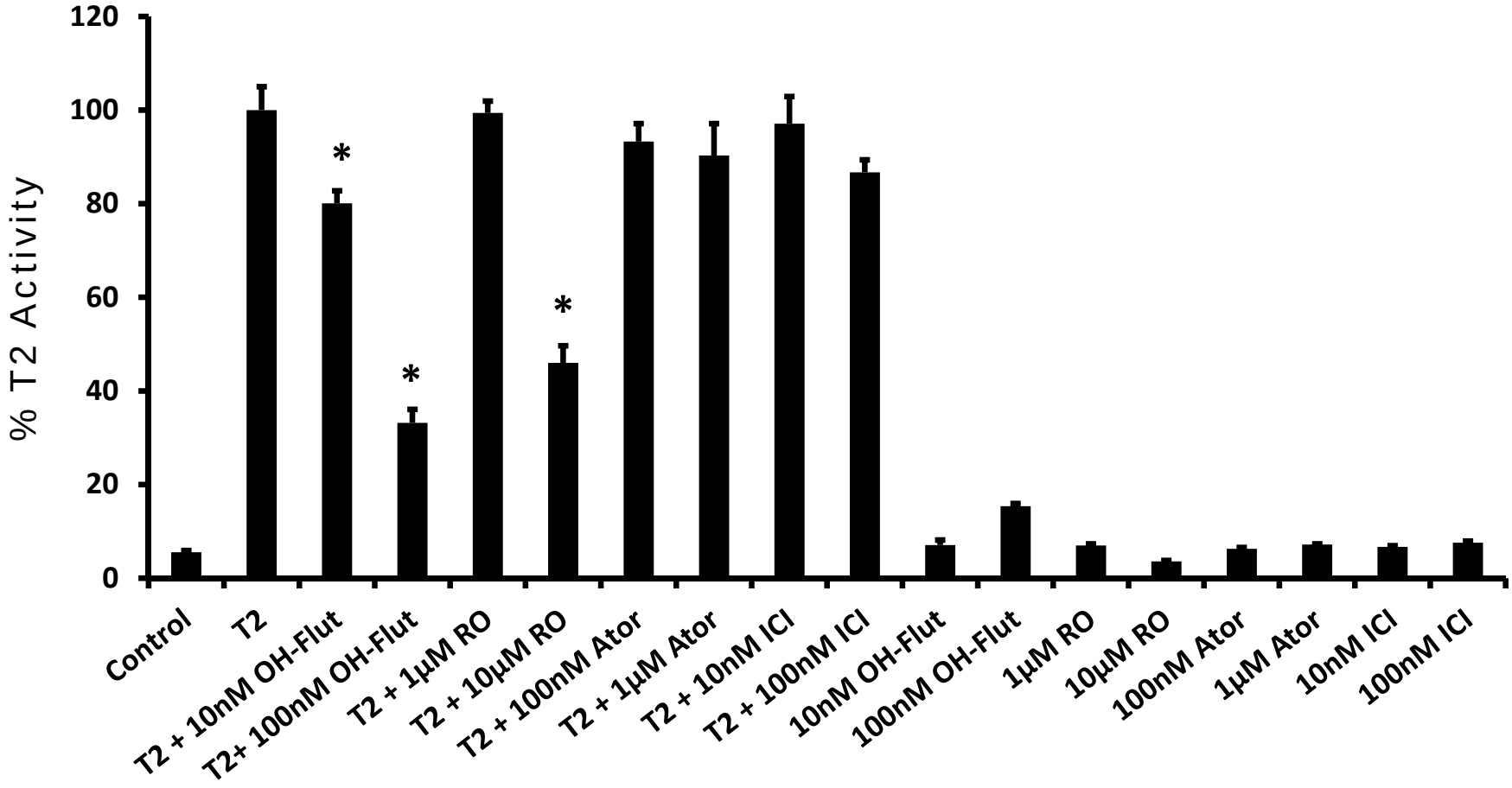


Figure 3D. Ator=Atorvastatin



<u>Figure 4:</u> RO significantly inhibits androgen-induced androgen receptor activity (luciferase). OH-Flut, hydroxyflutamide, an antagonist for androgen receptor; ICI= ICI 182,780, an antagonist for estrogen receptor; Ator, Atorvastatin, a statin used for lowering cholesterol



<u>Figure 5.</u> RO and other compounds tested do not show toxicity effects at the concentrations used. Sta= Staurosporine, an agent that induces apoptosis and kills cells.

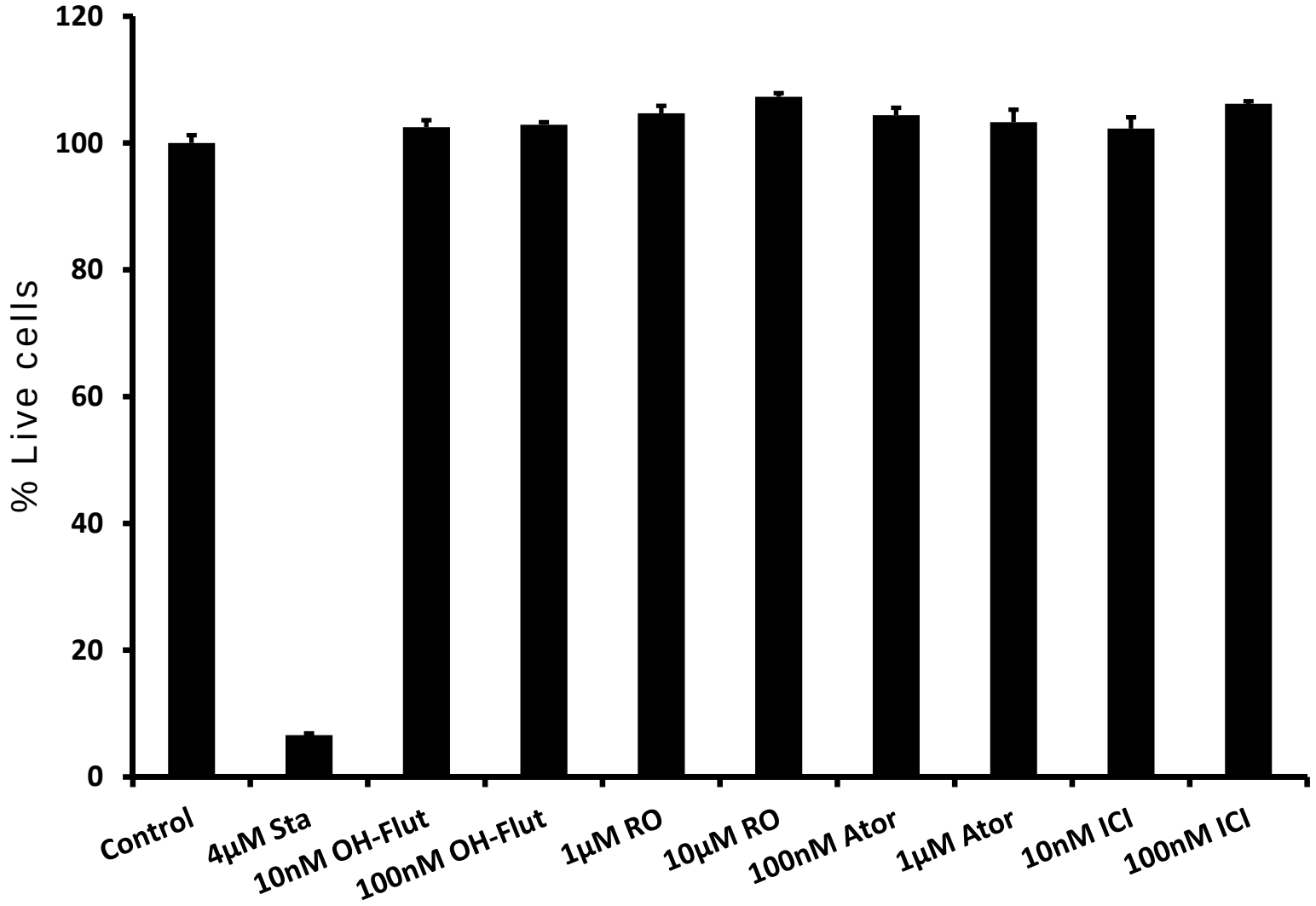


Figure 6A: RO inhibits estradiol –induced progesterone receptor expression in breast cancer cells. E2=Estradiol

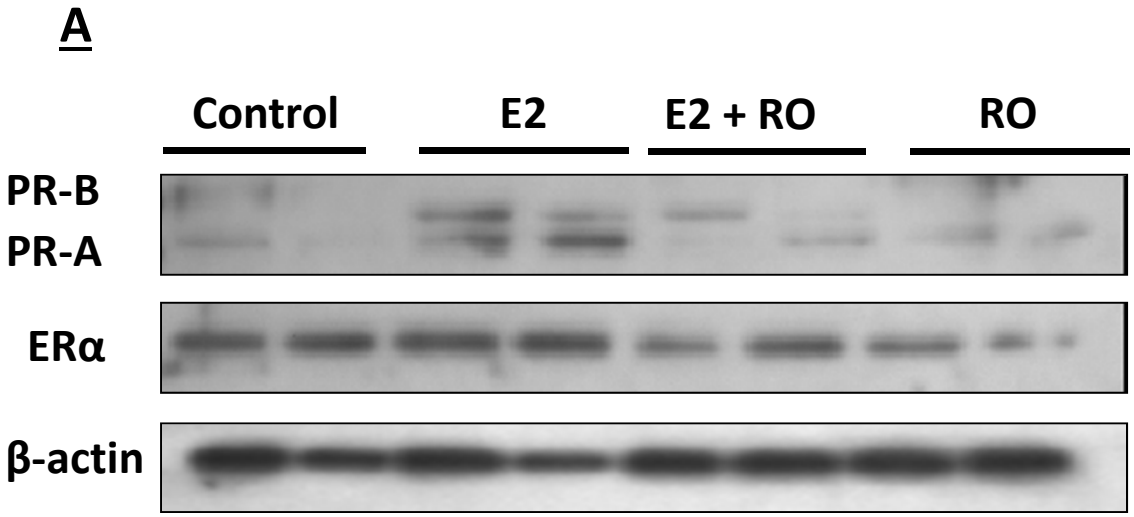


Figure 6B: Relative estrogen receptor mRNA expression in human breast cancer cells

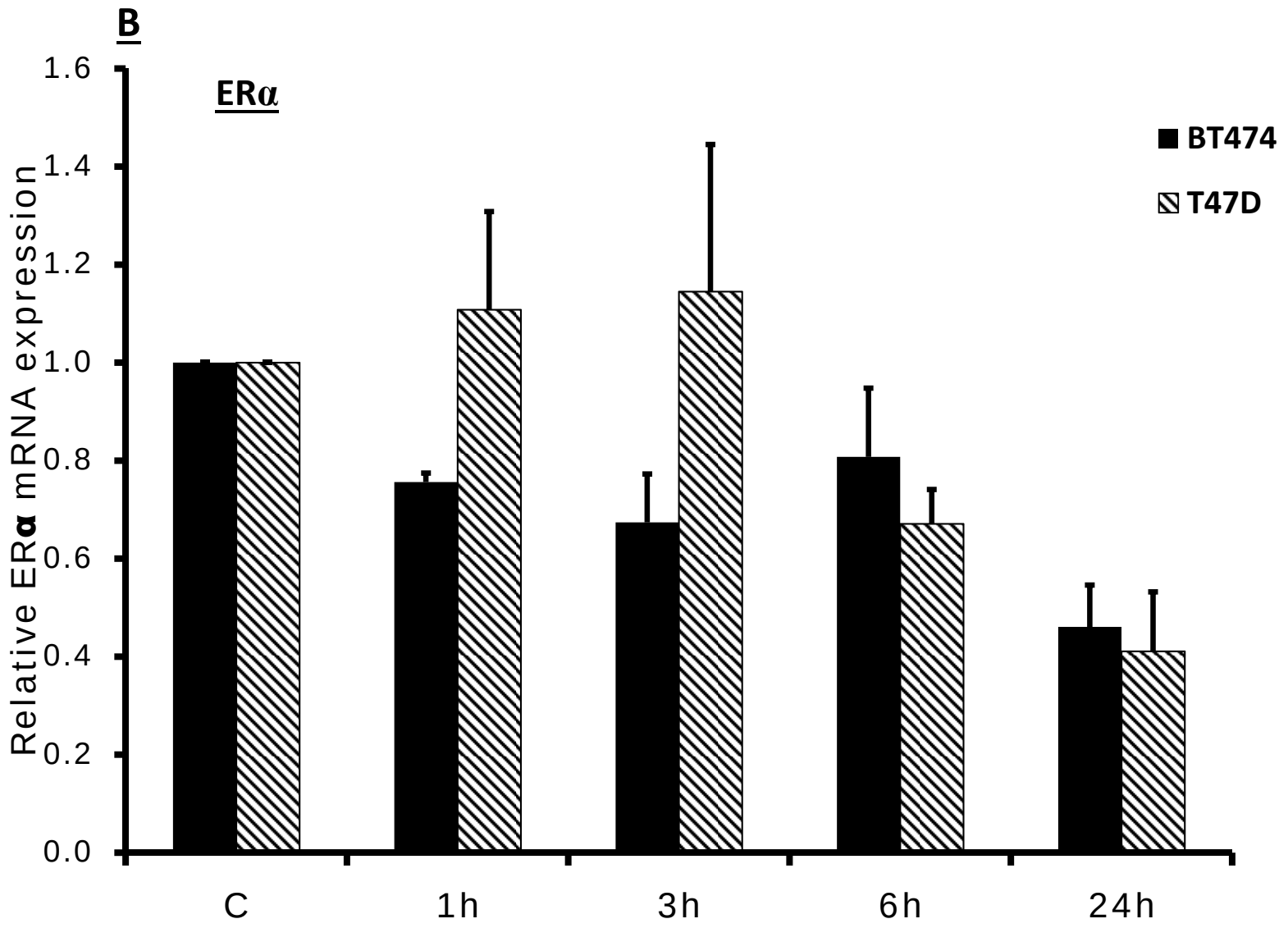


Fig 7A: Effect of 4-OH-Tamoxifen monotherapy on viability of different breast cancer cell lines. Note that sensitivity of MDA-MB-231 were less sensitive to tamoxifen.

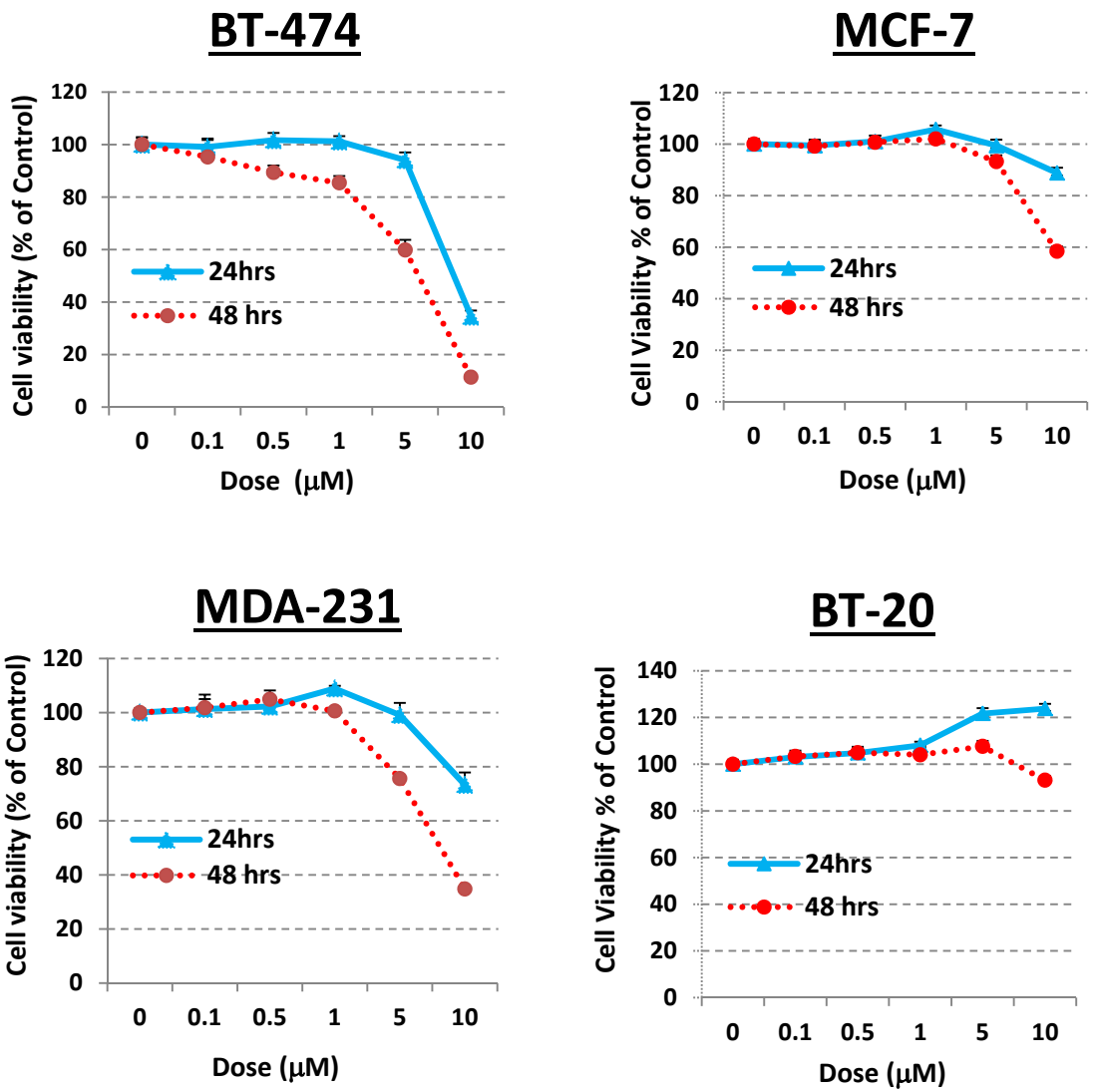
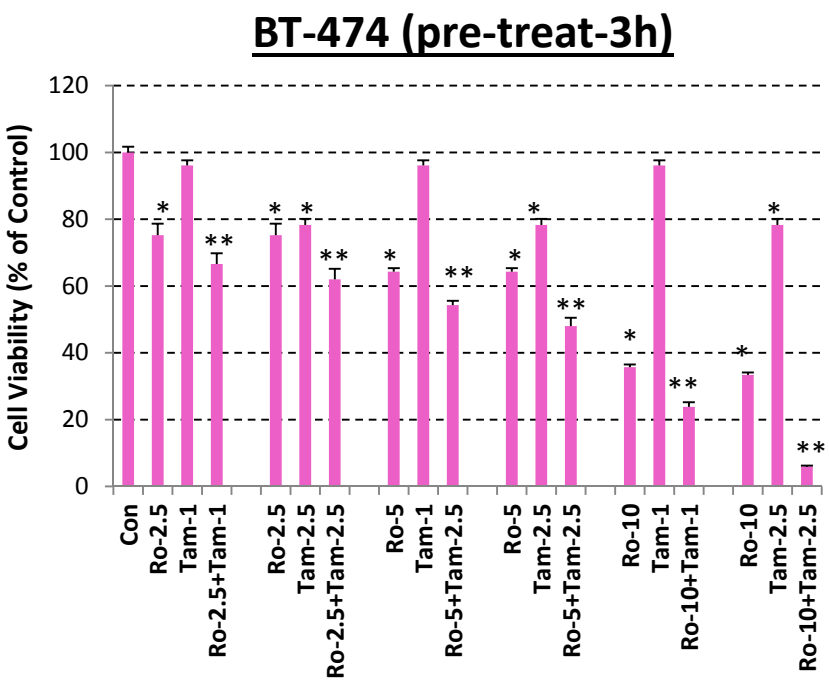


Fig 7B: Effect of combination therapy using Ro 48-8071 plus Tamoxifen on viability of BT-474 and MDA-MB-231 cells. Cells were pre-treated with RO to induce estrogen receptor beta. Comparison with Fig 7A shows that combination therapy is extremely effective against these cell lines. Concentrations are in μM. *, significantly different from control group, ** significantly different from other groups (ANOVA).



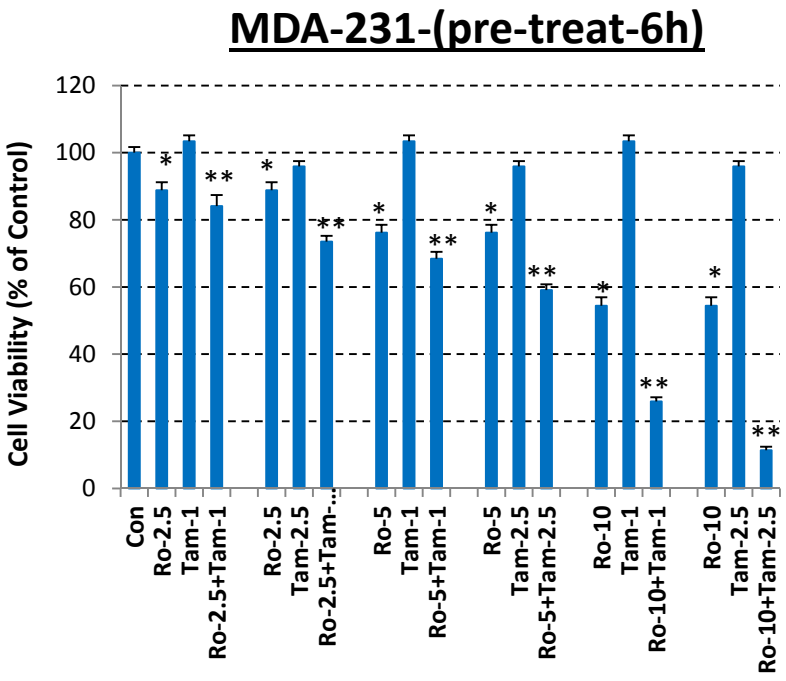


Fig 8A: Effect of Liquiritigenin, an estrogen receptor beta agonist, on viability of different breast cancer cell lines.

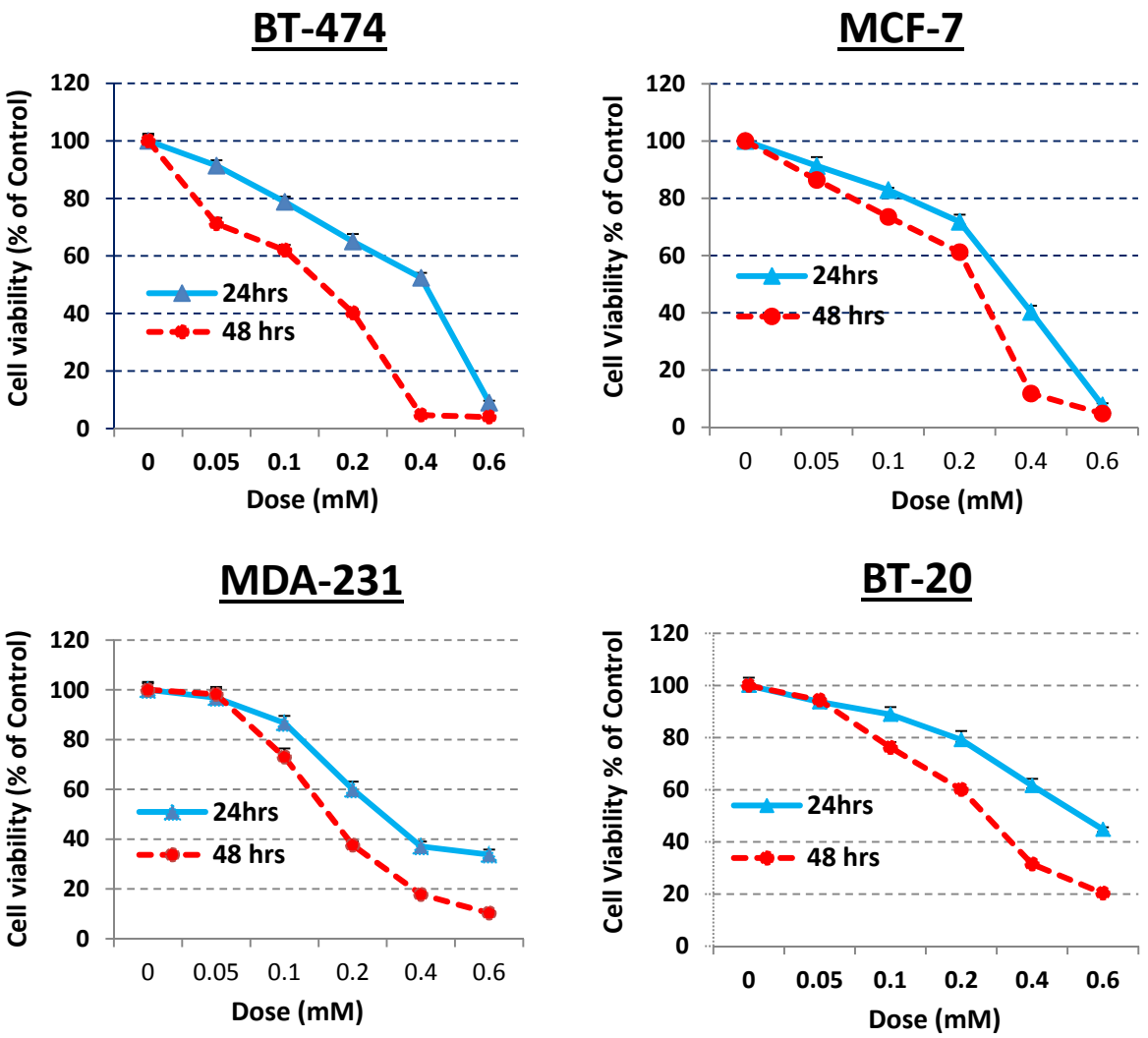


Fig 8B: Effect of Ro 48-8071 plus Liquiritigenin on growth of estrogen receptor alpha positive breast cancer cell lines (treatment 24 h). Cells were pre-treated with RO for 3-6 h to induce estrogen receptor beta and then RO was added. Figure shows that combination therapy is extremely effective against these cell lines. Concentrations are in μM. *, significantly different from control group, ** significantly different from other groups (ANOVA).

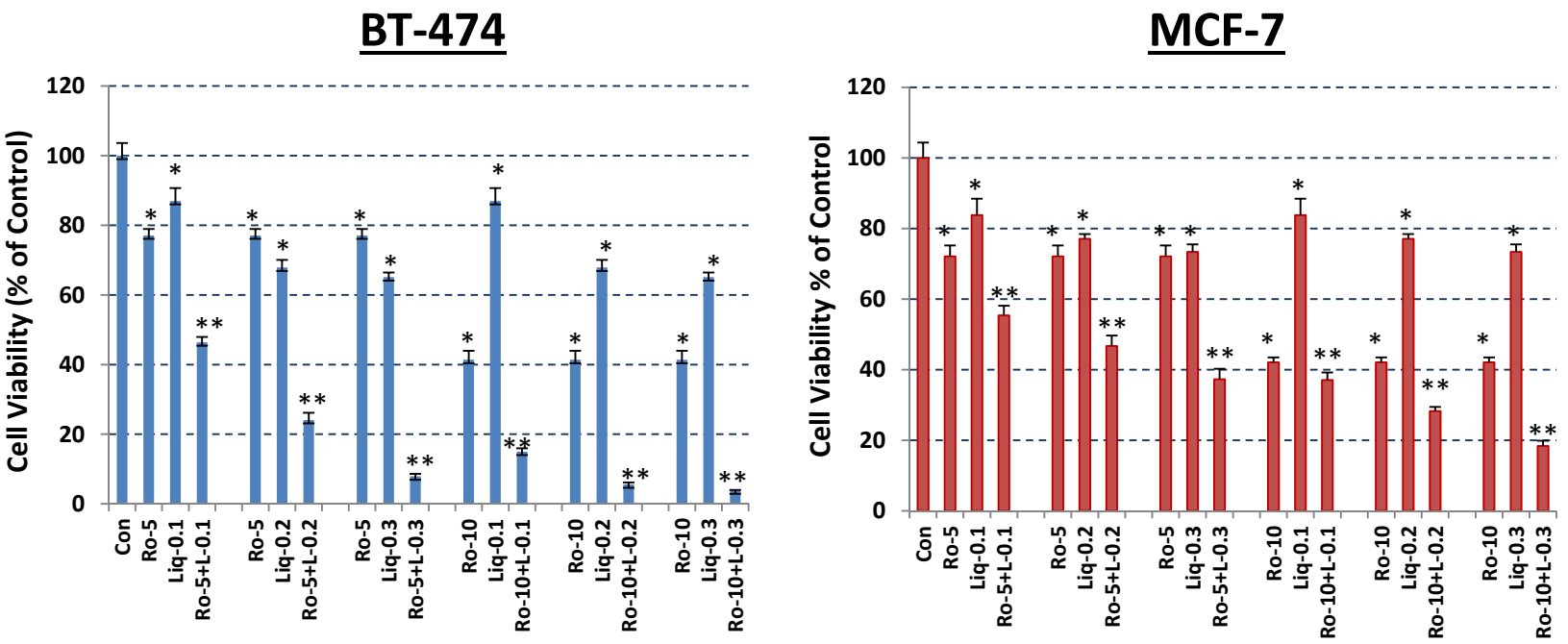


Fig 8C: Effect of Ro 48-8071 plus Liquiritigenin on growth of estrogen receptor alpha negative breast cancer cell lines. Cells were pre-treated with RO to induce estrogen receptor beta and then treatment was continued for another 18 h. Concentrations are in μM. Combination therapy was extremly effective as shown in the figure below. *, significantly different from control group, ** significantly different from other groups (ANOVA).

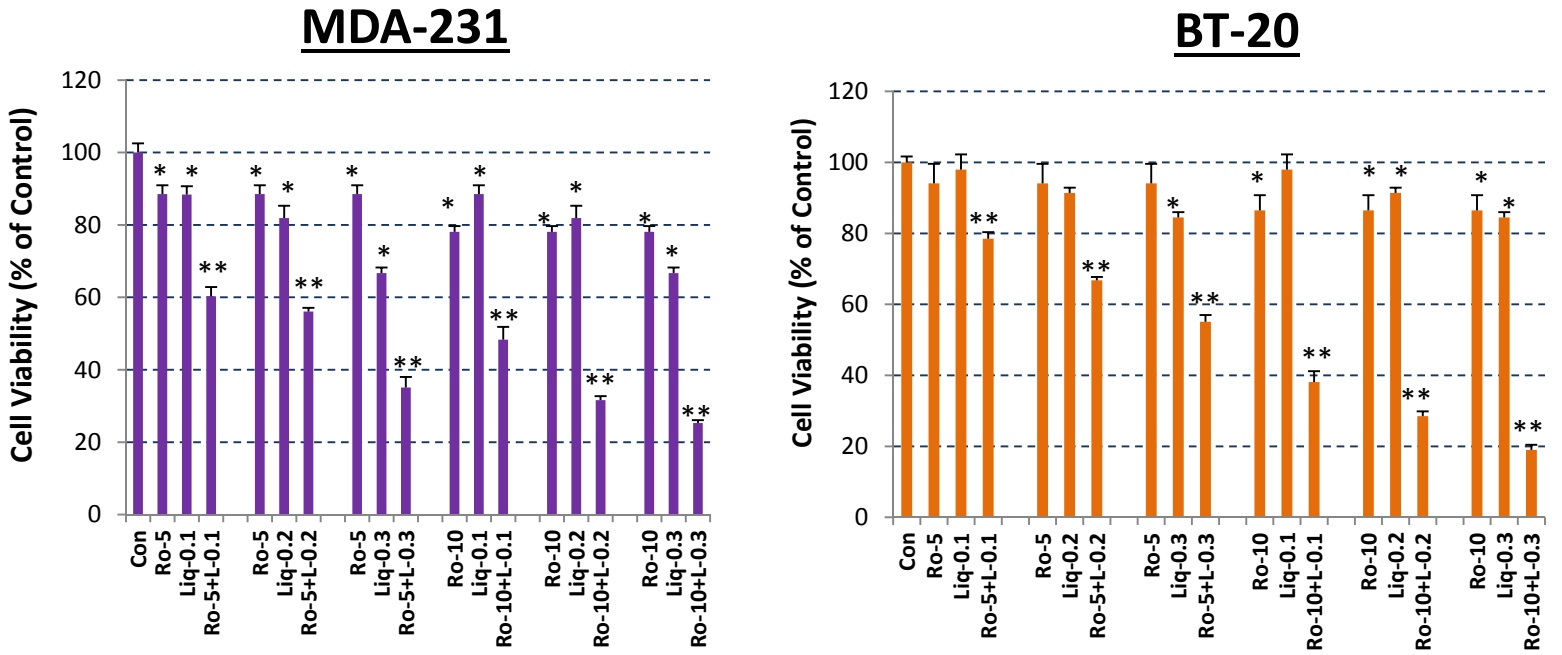


Fig 9A: Effect of Ro 48-8071 combined with Liquiritigenin on in vivo growth of BT-474 xenografts. Treatment was with RO 10 mg/kg iv + Liquiritigenin 20 mg/kg ip over time period indicated. *, significantly different from control group; **, significantly different from all other groups.

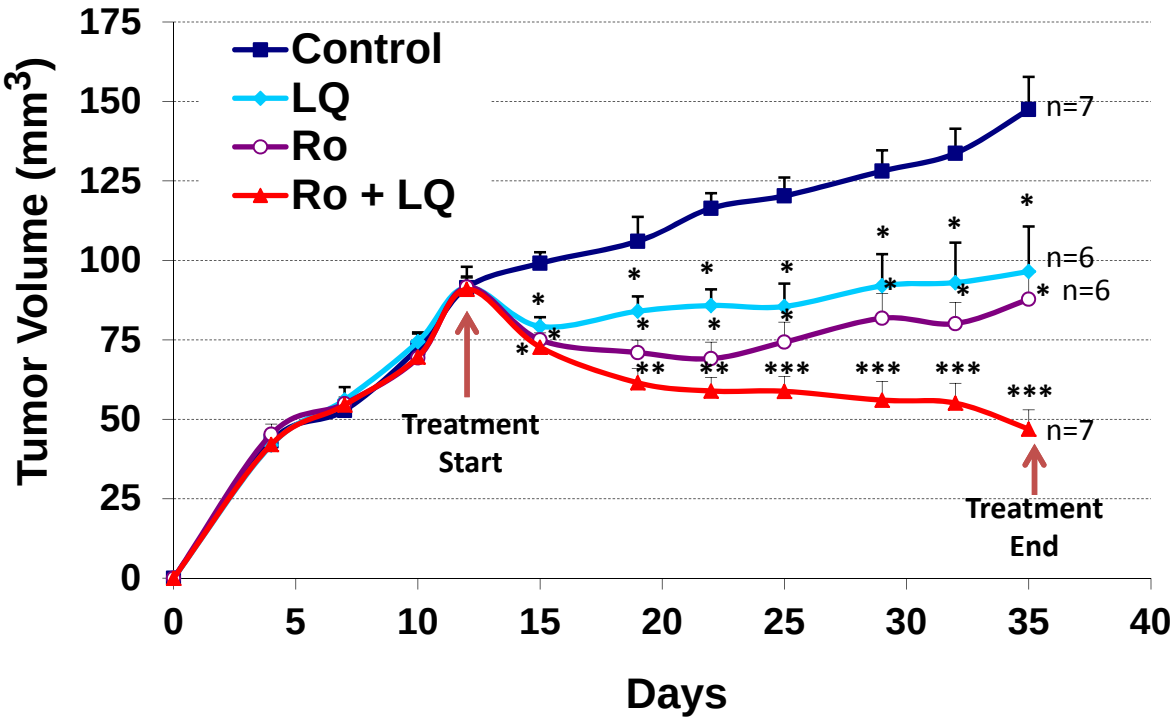


Fig 9B: Animal weight during drug treatment shown in Fig 9A.

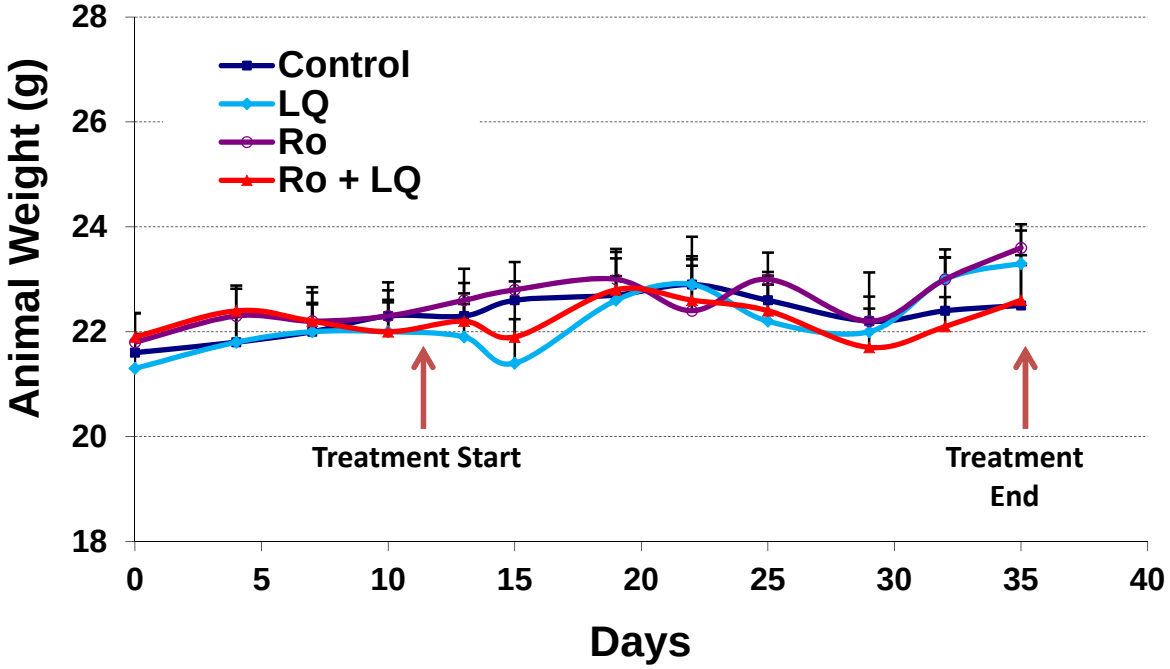


Fig 9C: Effect of RO 48-8071 combined with Liquiritigenin on clearance of BT-474 tumors in vivo.

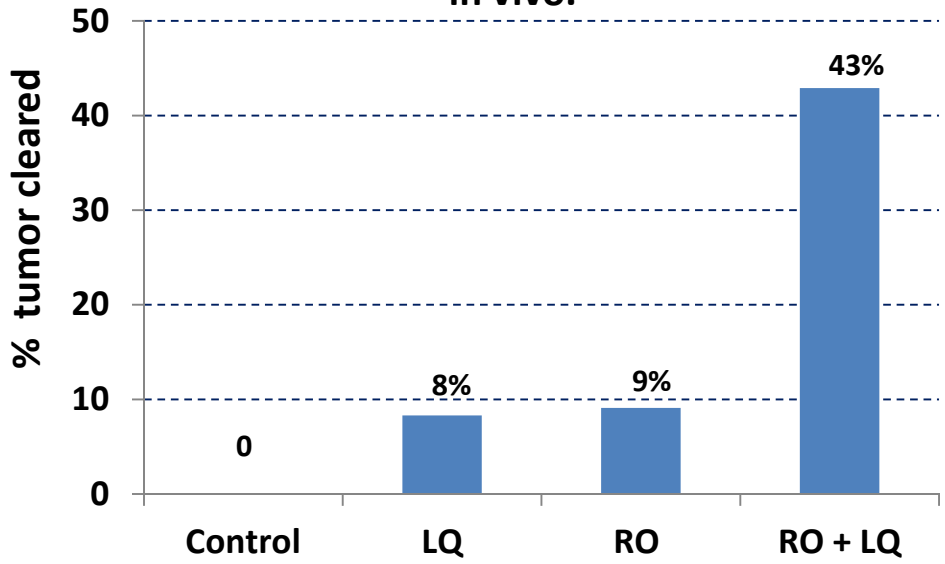


Fig 10A: Effect of Ro 48-8071 combined with Liquiritigenin on in vivo growth of MDA-MB-231 xenografts. Treatment was with RO 10 mg/kg iv + Liquiritigenin 20 mg/kg ip over time period indicated. *, significantly different from control; ** significantly different from all other groups (ANOVA)

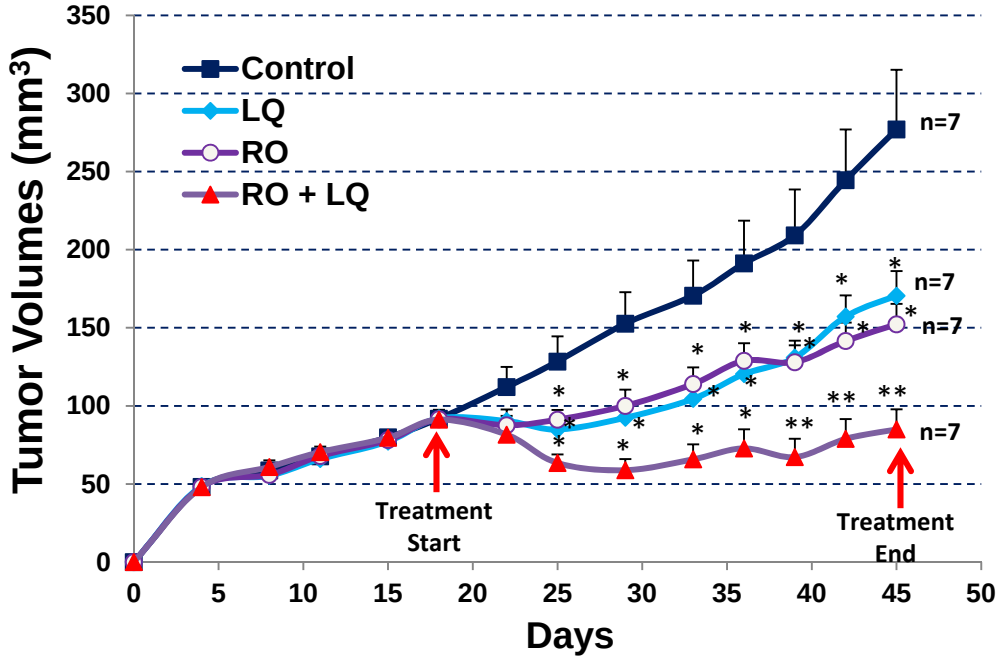


Fig 10B: Animal weight during drug treatment shown in Fig 10A

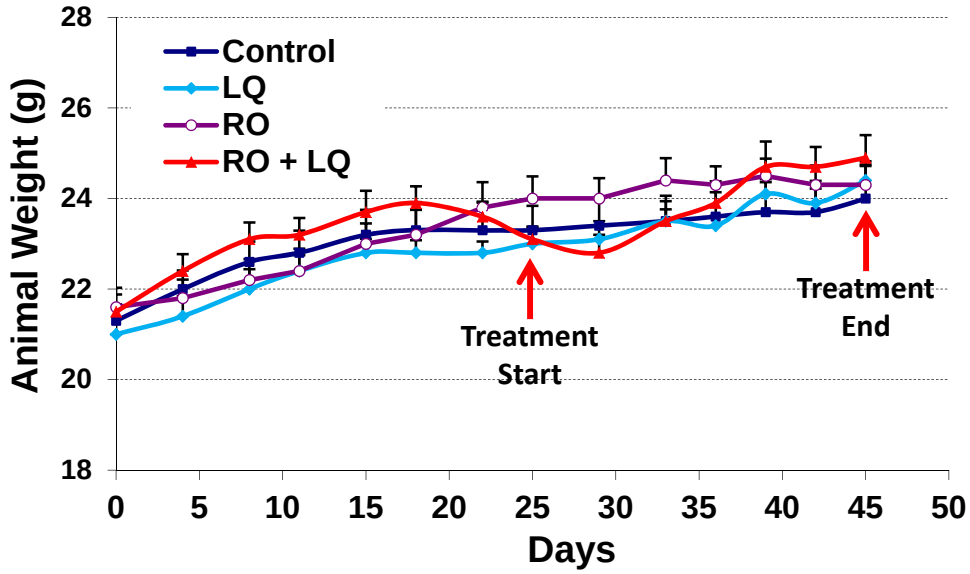


Fig 11A: Role of Ro-48-8071 (Ro) alone and in combination with Liquiritigenin (LQ) on promoting apoptosis (arrows) in BT-474 xenografts. Tumors were derived from experiment shown in Fig 9A. *, significantly different from control group; **, significantly different from RO and LQ groups.

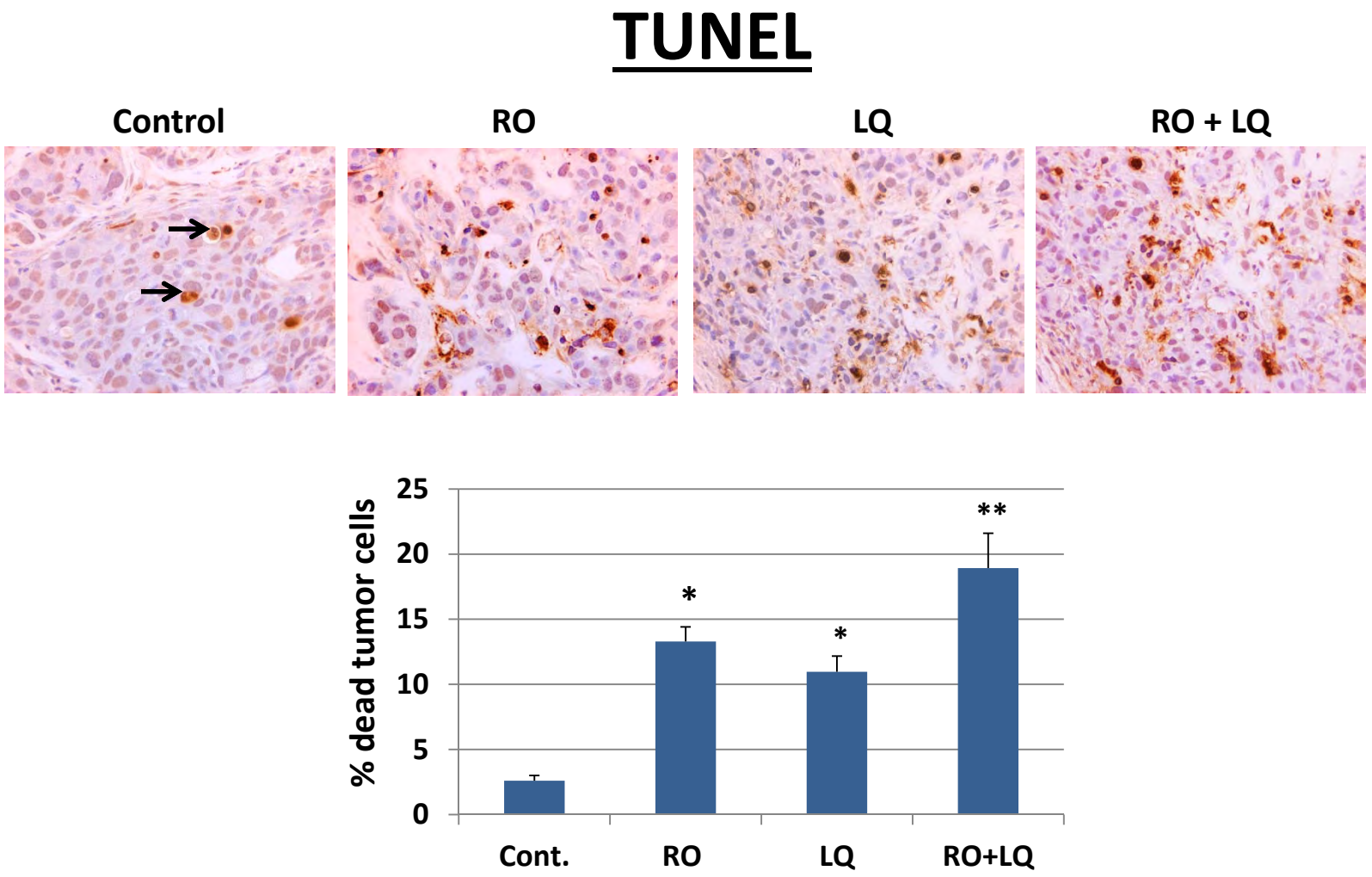


Fig 11B: Role of Ro-48-8071 (Ro) alone and in combination with Liquiritigenin (LQ) on reducing VEGF levels in BT-474 xenografts. Tumors were derived from experiment shown in Fig 9A. *, significantly different from control group; **, significantly different from RO and LQ groups.

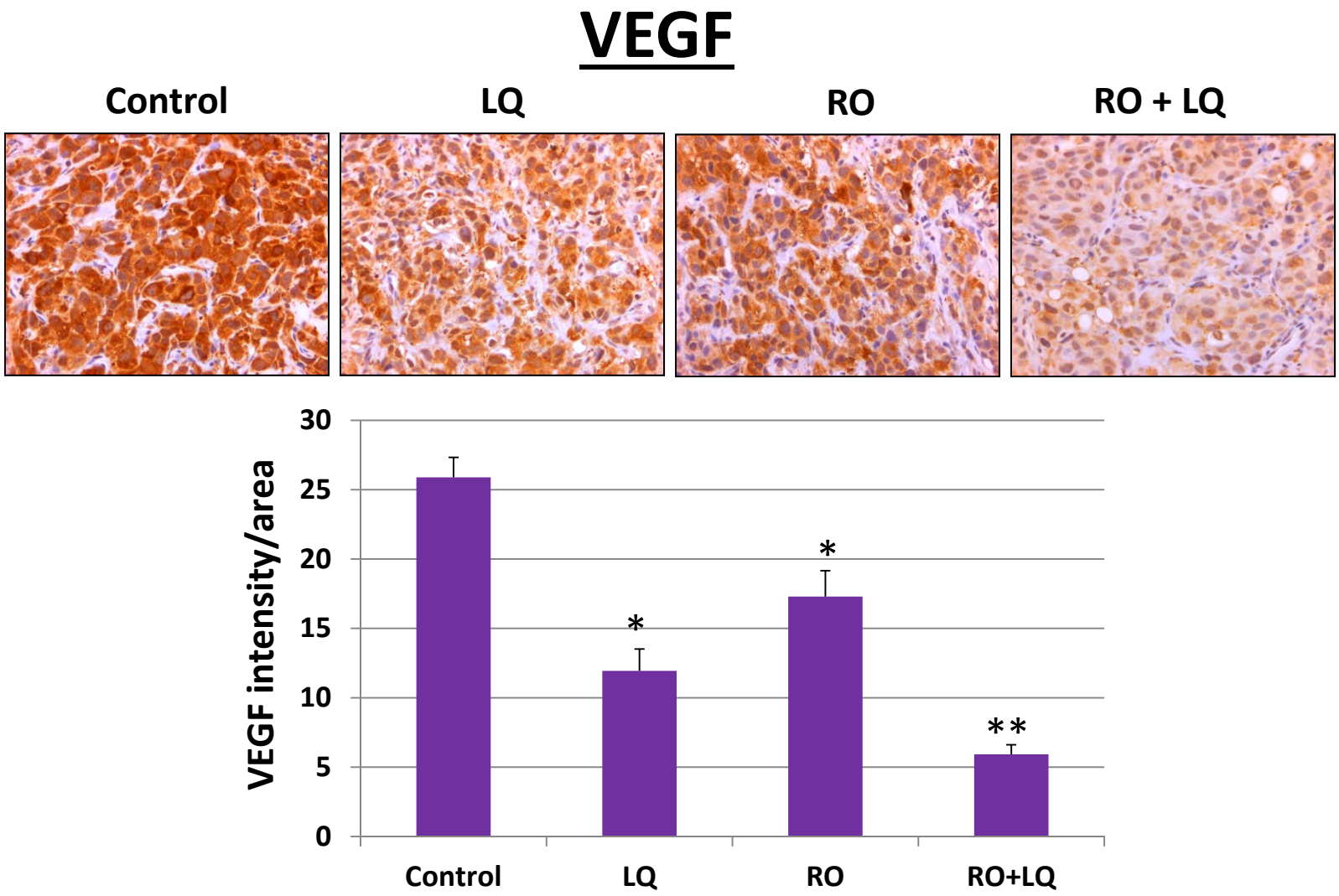
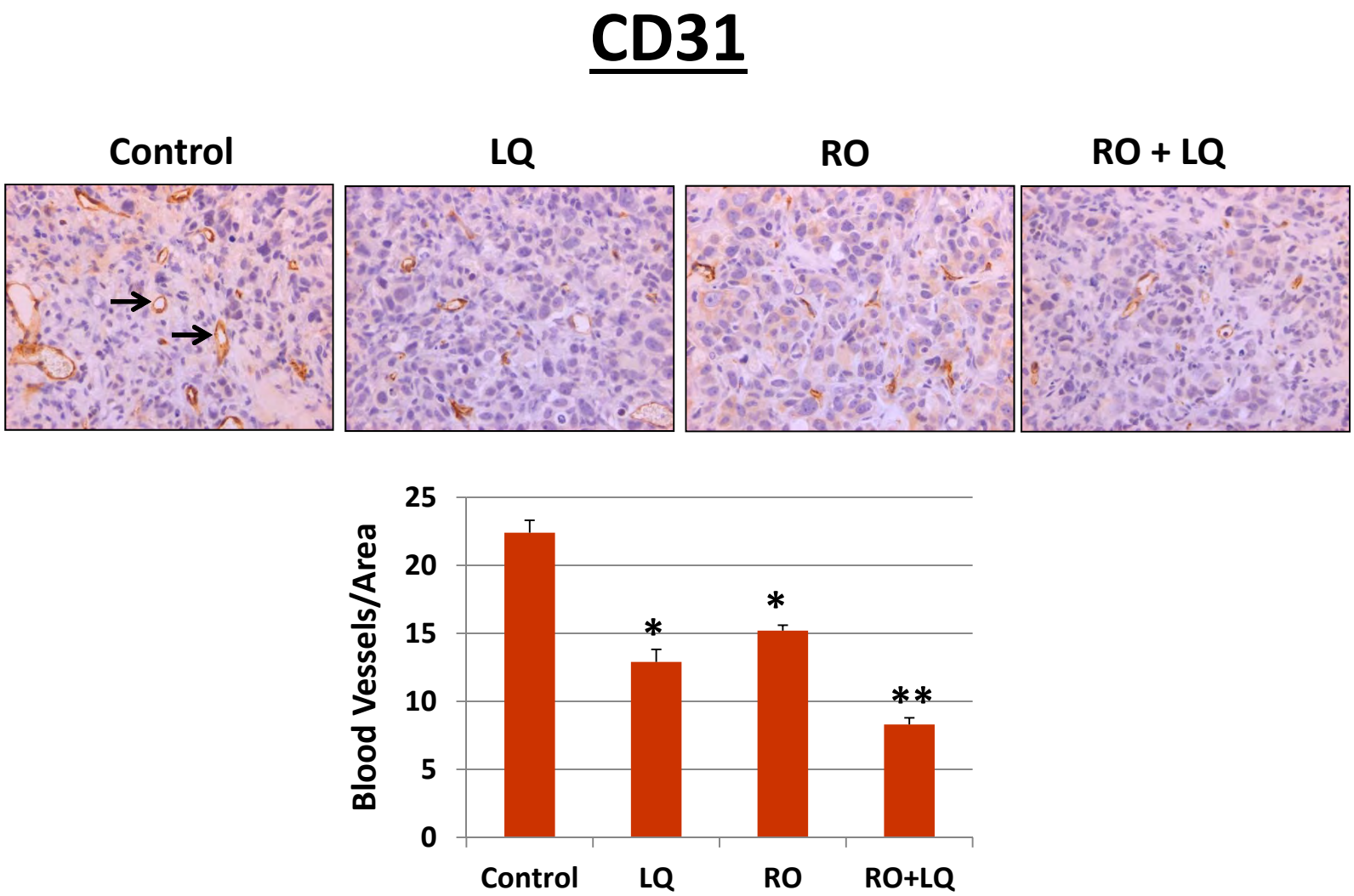


Fig 11C: Role of Ro-48-8071 (Ro) alone and in combination with Liquiritigenin (LQ) on reducing blood vessel density (arrows) in BT-474 xenografts. Tumors were derived from experiment shown in Fig 9A. *, significantly different from control group; **, significantly different from RO and LQ groups.



Breast Cancer Res Treat (2014) 146:51–62
DOI 10.1007/s10549-014-2996-5

PRECLINICAL STUDY

Cholesterol biosynthesis inhibitors as potent novel anti-cancer agents: suppression of hormone-dependent breast cancer by the oxidosqualene cyclase inhibitor RO 48-8071

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Received: 6 March 2014 / Accepted: 8 May 2014 / Published online: 31 May 2014

Abstract In most human breast cancers, tumor cell proliferation is estrogen dependent. Although hormone-responsive tumors initially respond to anti-estrogen therapies, most of them eventually develop resistance. Our goal was to identify alternative targets that might be regulated to control breast cancer progression. Sulforhodamine B assay was used to measure the viability of cultured human breast cancer cell lines exposed to various inhibitors. Protein expression in whole-cell extracts was determined by Western blotting. BT-474 tumor xenografts in nude mice were used for in vivo studies of tumor progression. RO 48-8071 ([4′-[6-(Allylmethylamino)hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate]; RO), a small-molecule inhibitor of oxidosqualene cyclase (OSC, a key enzyme in cholesterol biosynthesis), potently reduced breast cancer cell viability. In vitro exposure of estrogen receptor (ER)-positive human breast cancer cells to pharmacological levels of RO or a dose close to the IC_{50} for OSC (nM) reduced cell viability. Administration of RO to mice with BT-474 tumor xenografts prevented tumor growth, with no apparent toxicity. RO degraded ERα while concomitantly inducing the anti-proliferative protein ERβ. Two other cholesterol-lowering drugs, Fluvastatin and Simvastatin, were less effective in reducing breast cancer cell viability and were found not to induce ERβ. ERβ inhibition or knockdown prevented RO-dependent loss of cell viability. Importantly, RO had no effect on the viability of normal human mammary cells. RO is a potent inhibitor of hormone-dependent human breast cancer cell proliferation. The anti-tumor properties of RO appear to be in part due to an off-target effect that increases the ratio of ERβ/ERα in breast cancer cells.

Keywords Breast cancer · Tumor progression · Cholesterol biosynthesis inhibitors · Estrogen receptor

Abbreviations

E	Estrogen
ER	Estrogen receptor
PR	Progesterone receptor
OSC	Oxidosqualene cyclase
RO	RO 48-8071 ([4′-[6-(Allylmethylamino) hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate])
FBS	Fetal bovine serum
SRB	Sulforhodamine B
PI	Propidium iodide

Electronic supplementary material The online version of this article (doi:10.1007/s10549-014-2996-5) contains supplementary material, which is available to authorized users.

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sc	Subcutaneous
iv	Intravenous
PBS	Phosphate-buffered saline
TBS-T	Tris-buffered saline containing 0.1 % Tween 20
ANOVA	Analysis of variance
SE	Standard error
DPN	2,3-bis(4-Hydroxyphenyl)-propionitrile
PHTPP	4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol
FACS	Fluorescence-activated cell sorting

Introduction

Estrogens (E) are essential steroid hormones that regulate sexual development and reproductive functions in humans. The diverse biological effects of E are mediated by the specific estrogen receptors (ER) ERα and ERβ [1–3]. Almost 70 % of human breast tumors express both ER and progesterone receptor (PR) and proliferate in response to the respective hormone [4–6]. At the cellular level, E and progestins stimulate cell proliferation and metastasis [4–7], promote angiogenesis [8], inhibit cell death [9, 10], and increase the risk of breast cancer in post-menopausal women on hormone replacement therapy [11–14]. ERα-positive breast cancers are usually treated with anti-estrogens and aromatase inhibitors, but resistance to these agents invariably develops during the course of therapy; these drug-resistant tumors then proliferate more aggressively than the drug-sensitive tumors from which they arose [15, 16]. Therefore, novel and more effective treatment strategies that could target ERs in hormone-dependent breast cancer are urgently needed.

Enzymes in the cholesterol biosynthetic pathway are attractive therapeutic targets for hormone-dependent breast cancer, because cholesterol serves as the metabolic precursor of endogenous steroid hormones, including those found in tumors [17, 18]. In addition, breast cancer cells have the capacity to synthesize cholesterol, and it is possible that endogenously produced cholesterol could contribute to the development of anti-hormone resistance [18, 19]. Statins, which are the most commonly used class of cholesterol-lowering drug, inhibit HMG-CoA reductase, an enzyme in the cholesterol biosynthetic pathway; however, certain undesirable side effects limit their long-term use for cancer therapy [20]. 2,3-Oxidosqualene cyclase (OSC) is an enzyme that acts downstream of HMG-CoA reductase to convert 2,3-monoepoxysqualene to lanosterol (a key step in the biosynthesis of cholesterol) [21–23]. While testing small-molecule inhibitors of OSC, we identified RO 48-8071 ([4′-[6-(Allylmethylamino)hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate] (RO) [21–23] as a potent suppressor of breast tumor cell viability [24]. In the present study, we describe the anti-tumor effects of RO on ERα-positive tumors, both in vitro and in vivo. We observed that in addition to its recognized properties, RO also had off-target effects, degrading ERα while concomitantly inducing ERβ, the latter of which has been shown to block proliferation of breast cancer cells [25–28] and suppress tumor angiogenesis [29]. Consistent with these findings, we found that the anti-proliferative effects of RO were blocked by an ERβ-specific antagonist and ERβ-targeted siRNA. RO also induced apoptosis of breast cancer cells. Thus, RO exhibits unique anti-tumor properties, making it an exciting candidate compound for clinical management of breast cancer progression when used as mono-therapy and potentially in combination with ERβ-specific ligands.

Materials and methods

Cell lines and culture

ERα-positive breast cancer cell lines and normal mammary cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in phenol red-free DMEM:F12 medium (Invitrogen Corporation & Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

Reagents

RO 48-8071, Fluvastatin, Simvastatin, ICI 182,780, and U1866A were purchased from Sigma-Aldrich; RO analogs were provided by Roche Pharmaceuticals (Basel, Switzerland) and were synthesized as previously described [22, 30]. MG-132 was from Calbiochem; 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were from Tocris Biosciences. Sixty-day release pellets containing 17-β-estradiol (1.7 mg) or placebo were obtained from Innovative Research of America (Sarasota, FL, USA). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), as were human ER-β-siRNA (sc-35325) and scrambled siRNA (sc-37007). Lipofectamine™, RNAiMAX, and Opti-MEM medium were obtained from Invitrogen Corporation & Life Technologies. RNAZol for RNA isolation was purchased from Molecular Research (Cincinnati, OH, USA).

Cell viability assay

The sulforhodamine B (SRB) assay was used to measure cell viability, as previously described by us [31].

Cell apoptosis and death assay

Cells were analyzed for apoptosis using the Annexin V-FITC Apoptosis Detection Kit (Biovision Research Products, Mountain View, CA, USA) as previously described [32].

In vivo breast tumor growth inhibition assays

All animal experiments were approved by the Institutional Review Committee. Female athymic nude mice (nu/nu, Foxn1), 5 to 6 weeks old and weighing 20–22 g, were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). Mice were implanted subcutaneously (sc) with pellets containing either 17-β-estradiol (1.7 mg/pellet, 60-day release) or placebo prior to inoculation of BT-474 breast cancer cells as previously described by us [33]. Tumor volumes were measured as described previously [33], and drug treatment was started when tumor volumes reached approximately 100 mm^3. Mice treated with RO received 5 or 10 mg/kg by intravenous (iv) injection of a 0.1 ml solution into the tail vein daily for 5 days, followed by an injection every other day for five additional treatments and then a final injection 2 h prior to sacrifice. Control mice received the same volume of phosphate-buffered saline (PBS) on the same schedule. Animals were weighed twice weekly throughout the study.

Tumors were collected following the last injection and processed for immunohistochemical analysis of ERα and ERβ as described previously [33, 34]. Quantitation of immunolabeled signal was achieved using a morphometric analysis program (FoveaPro 3.0, Reindeer graphics), on images photographed at 20× magnification as described earlier [34]. 3–4 Tumors/groups were analyzed for ER signal, and 2–3 representative sections were collected from each tumor. Results are expressed as area in square pixels.

Western blots

Whole-cell extracts were prepared with a nuclear extraction TransAm kit (Active Motif, Carlsbad, CA, USA) as described previously, and Western blotting was carried out as previously described [30, 32, 33].

siRNA knockdown

ERβ siRNA transfection was conducted following the manufacturers protocol (Santa Cruz). The transfection medium used was Opti-MEM, and transfection reagent was Lipfectamine RNAiMAX. The day before transfection, cells were seeded in 6-well plates at a density of 8×10^4 cells/well with 10 % FBS DMEM:F12 medium. Cells were incubated for 24 h with siRNA, after which 1 ml fresh 10 % FBS DMEM:F12 medium was added to each well. Cells were then incubated for another 24–48 h prior to treatment with RO.

Statistical analysis

Differences between groups or among groups were tested, respectively, using one-way analysis of variance (ANOVA) with repeated measures over time. The assumption of the ANOVA was examined, and a nonparametric measure based on ranks was used if needed. Values are reported as mean ± SEM. When ANOVA indicated a significant effect (F-ratio, $P < 0.05$), the Student–Newman–Keuls multi-range test was used to compare the means of the individual groups. Statistical analyses were conducted using SigmaStat software, version 3.5. For immunohistochemical analysis, data were analyzed using Kruskal–Wallis ANOVA, followed by Tukey's procedure as a post hoc test. For all comparisons, $P < 0.05$ was regarded as statistically significant. Values are reported as mean ± SEM.

Results

OSC inhibitors reduce cell viability of ERα-positive breast cancer cells but not normal mammary cells

Using several ERα-positive breast cancer cells, we tested the ability of four OSC inhibitors to reduce cell viability (Fig. 1a). While all four compounds reduced cell viability, RO 48-8071 and RO 61-3479 most effectively reduced the viability of BT-474, T47-D, and MCF-7 cells in a time- and dose-dependent manner. We selected RO 48-8071 (referred to as RO from this point forward) as the lead compound for further studies. RO also effectively reduced cell viability of HCC-1428 and ZR-75 cells (Online Resource 1). The IC_{50} values for the cell lines tested ranged from approximately 6–15 μM in a 24–48 h SRB assay (Table 1). Because the affinity of RO for OSC is in the nM range [22, 23], we examined whether a range of low doses of RO would affect cell viability over an extended period of time (7-day assay) similar to the effects observed for higher doses over a 24-h period. We found that RO concentrations as low as 1 nM effectively reduced BT-474 and MCF-7 cell viability in 7-day assays (Fig. 1b). To determine whether RO specifically reduces cancer cell viability, leaving normal cells unaffected, we conducted studies using normal AG11132A

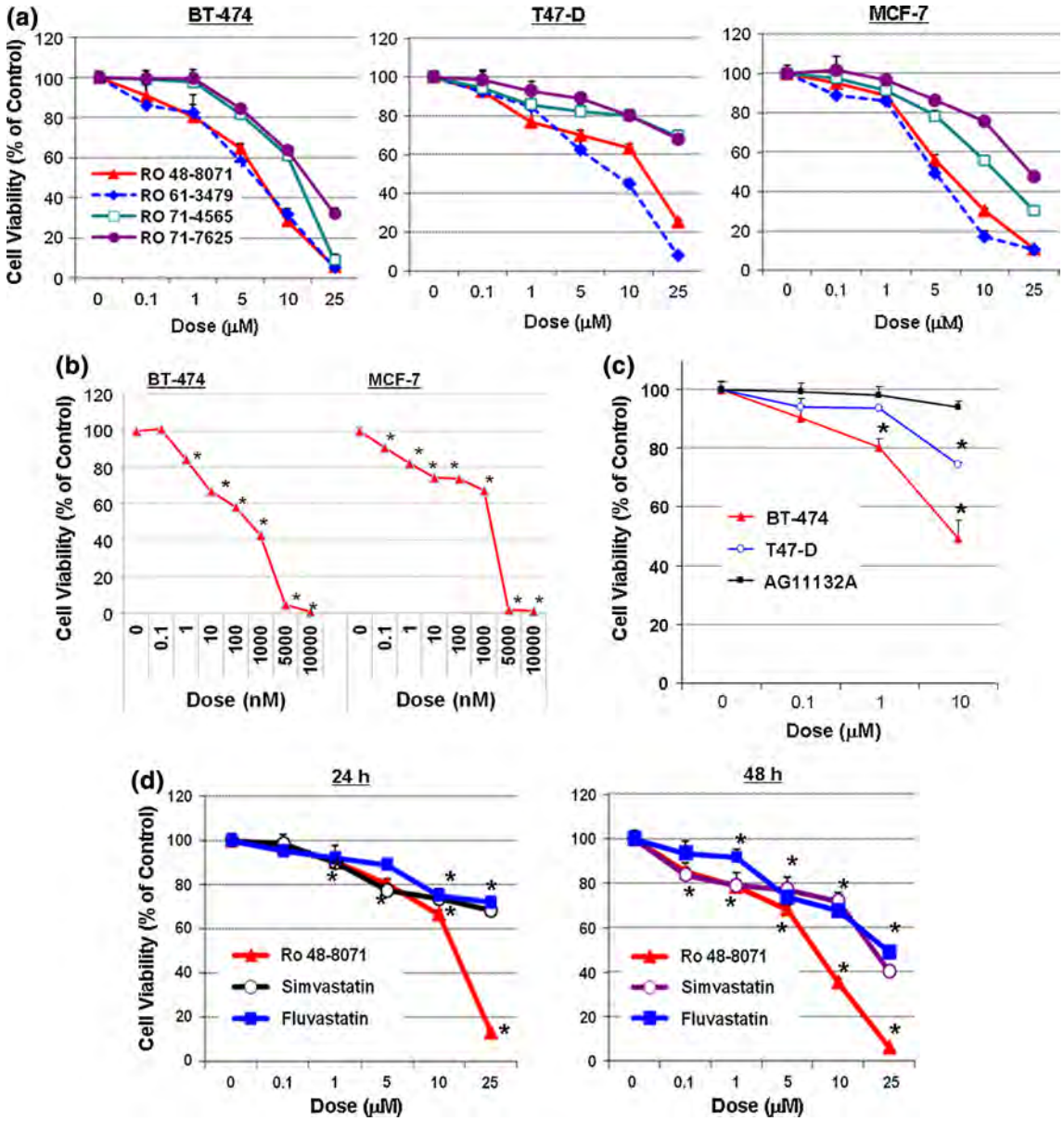

Fig. 1 OSC inhibitors reduce the viability of breast cancer cells but not normal mammary cells. **a** Breast cancer cells were incubated with pharmacological doses of indicated compounds for 48 h. **b** Breast cancer cells were incubated with low-dose (nM range) RO for 7 days. **c** Normal mammary cells (AG11132A) were treated with pharmacological doses of RO for 24 h and compared directly with the two cancer cell lines shown. **d** BT-474 breast cancer cells were treated with RO or the statins Simvastatin or Fluvastatin for 24 or 48 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control (set at 100 %) ($P < 0.05$ using ANOVA)

Table 1 IC_{-n} values of RO 48-8071 on breast cancer cell lines

Cell lines	IC_{50} (μM) (24 h)	IC_{50} (μM) (48 h)
BT-474	9.51 ± 0.05	6.06 ± 0.23
T47-D	11.53 ± 0.36	7.76 ± 0.29
MCF-7	12.32 ± 0.59	6.34 ± 0.34
HCC-1428	14.64 ± 0.42	11.58 ± 0.34
ZR-75	11.04 ± 0.29	7.63 ± 0.30

mammary cells. Concentrations of RO up to 10 μM reduced cancer cell viability, but had no effect on normal cells (Fig. 1c).

We also compared the ability of RO to reduce breast cancer cell viability with that of two other inhibitors of cholesterol biosynthesis (statins). The HMG-CoA reductase inhibitors Simvastatin and Fluvastatin also reduced cell viability; however, RO was more effective than either statins in 24- or 48-h assays (Fig. 1d).

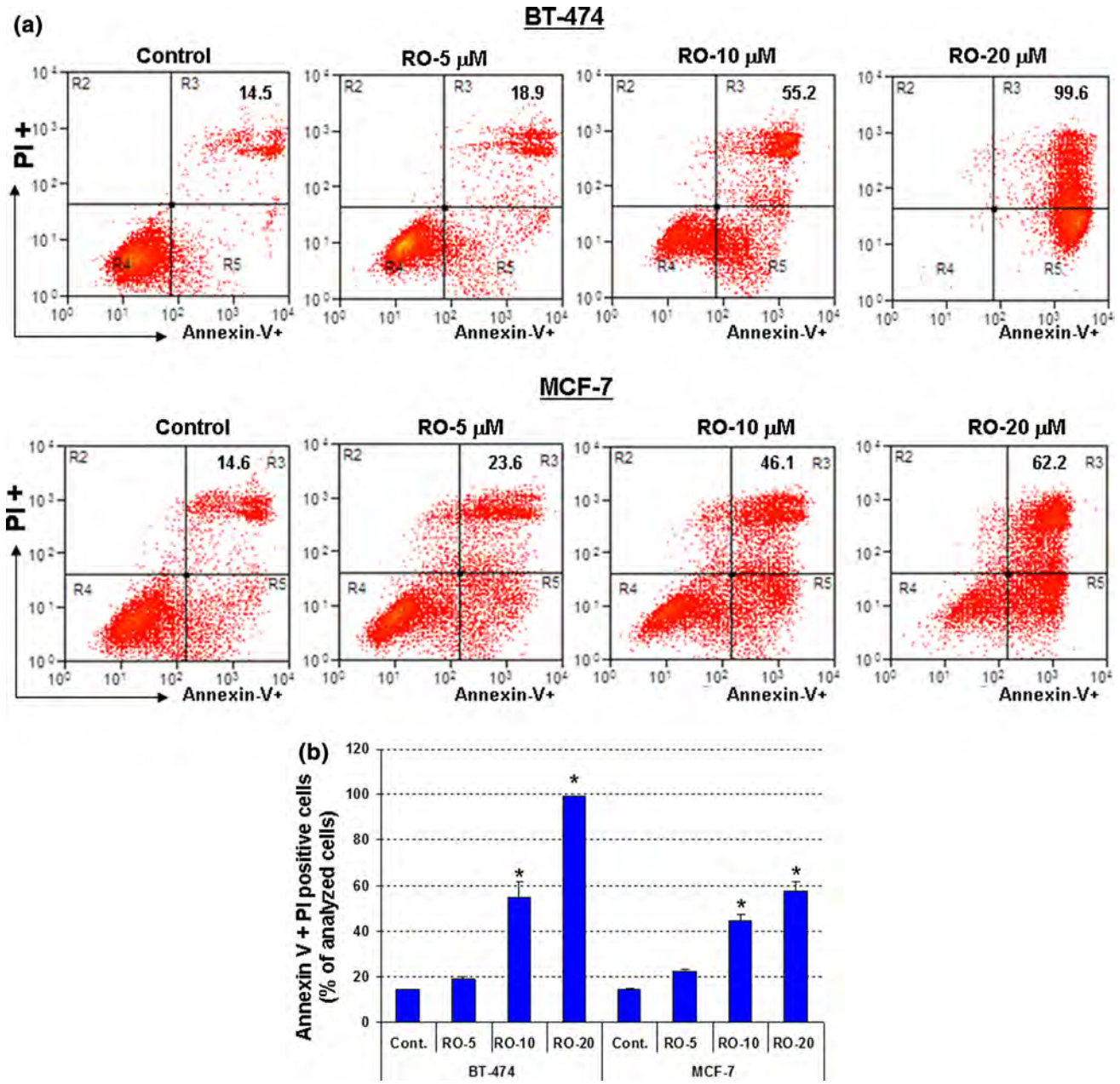

Fig. 2 RO induces apoptosis and cell death in breast cancer cells. **a** BT-474 and MCF-7 cells were seeded in 6-well plates overnight in 10 % FBS DMEM:F12 (1.5×10^5/well). After washing and replacement of media, cells were treated with 5, 10, or 20 μM RO or vehicle alone (control) for 24 h. Following treatment, cells were harvested and stained with annexin V-FITC and propidium iodide (PI). Fluorescence-activated cell sorting (FACS) analysis of 10,000 cells/sample was conducted. Quadrant R5 (*bottom right*) shows annexin V-positive (apoptotic) cells, and quadrant R3 (*top right*) shows annexin V-positive/PI-positive (dead) cells. **b** Quantitative data from FACS analysis. Values represent mean ± SEM ($n = 3$). *Significantly different from control ($P < 0.05$ using ANOVA)

RO induces apoptosis and cell death in breast cancer cells

In order to determine the mechanism by which RO reduced breast cancer cell viability, we treated BT-474 and MCF-7 cells for 24 h with 5, 10, or 20 μM RO. Cells were then collected, and the levels of apoptosis and cell death were determined. RO significantly induced apoptosis and cell death in both cell lines in a dose-dependent manner (Fig. 2a, b).

RO suppresses E-dependent proliferation of breast cancer cells in vitro and in vivo

Because E promotes proliferation of ERα-positive cells [1, 4], we examined whether RO reduces hormone-dependent

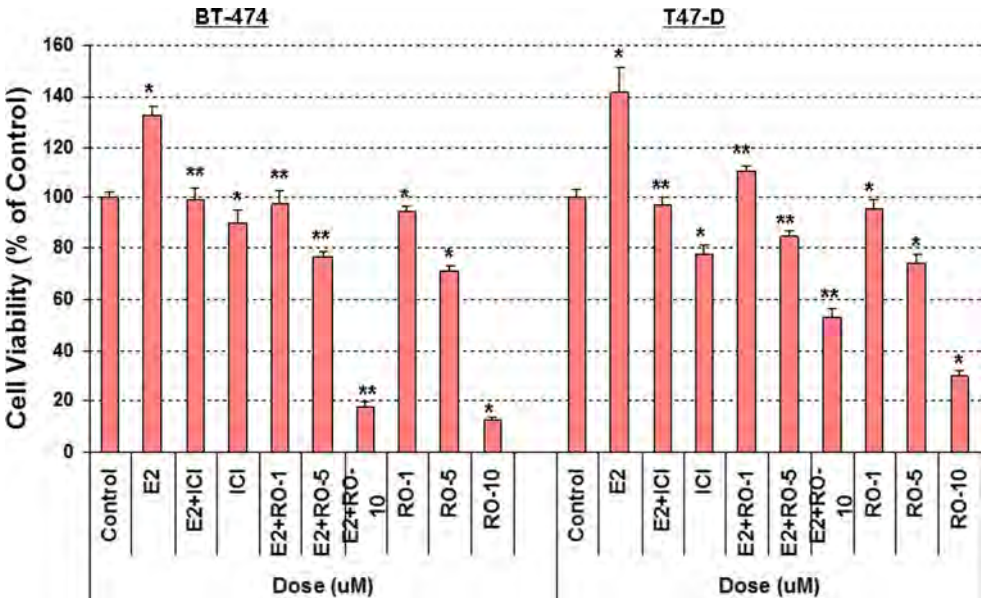

Fig. 3 RO suppresses Estradiol (E2)-induced proliferation of breast cancer cells. Breast cancer cells were treated with or without 10 nM E2 ± 1, 5, or 10 μM RO or 1 μM ICI 182,780 (ICI) for 24 h in 5 % charcoal stripped serum, after which cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control (set at 100 %); **significantly different from E2 ($P < 0.05$ using ANOVA)

proliferation of breast cancer cells. Using the anti-estrogen ICI 182,780 (which suppresses E-dependent cell proliferation [35, 36]) as our control ligand, we found that RO blocked E-dependent proliferation in four different breast cancer cell lines (Fig. 3 and data not shown for ZR-75 and MCF-7 cells). Furthermore, concentrations of RO that reduced E-induced cell proliferation also reduced cell viability in the absence of E.

Having demonstrated the effectiveness of RO in suppressing E-dependent breast cancer cell growth in vitro, we conducted studies to establish whether it had the same effect in vivo. We established estrogen-dependent BT-474 tumor xenografts in nude mice and began treatment with RO when the tumor volumes were approximately 100 mm^3. Compared with controls, the tumor burden of animals administered RO was significantly reduced (Fig. 4a). Furthermore, animal weights were unaffected by RO treatment, indicating that the compound was non-toxic at the dose administered (Fig. 4b). No changes in blood chemistry or evidence of cataracts was observed, as determined by CBW, Head Pathologist IDDEX RADIL (data not shown).

In order to determine the effects of RO on levels of ERα and ERβ protein expression in xenografts, we conducted immunohistochemical analysis of sections obtained from tumors collected at the end point in Fig. 4a. RO treatment resulted in significantly reduced levels of ERα within tumor tissue; however, ERβ was more resilient to depletion (Fig. 4c). While there was a trend toward elevated levels of ERβ in animals receiving 5 and 10 mg/kg, significance was attained in only the 10 mg/kg treatment group.

RO reduces levels of ERα protein and increases levels of ERβ protein in breast cancer cells in vitro

Because RO prevented E-induced cell proliferation and caused a loss of ERα protein, we determined whether it affected levels of ERα and ERβ in breast cancer cells in vitro. Pharmacological levels (25 μM) of RO reduced ERα in three breast cancer cell lines in a time-dependent manner. RO was most effective against BT-474 cells; most of the receptor was lost after just 3 h of exposure in these cells, compared with 6 h for other cell lines (Fig. 5a, upper panel). The loss of ERα following treatment with RO for 3 h (BT-474) or 6 h (T47-D and MCF-7) was dose-dependent (1–25 μM) (Fig. 5a, lower panel). Using BT-474 cells, we tested whether loss of ERα was due to proteasomal degradation. We found that this RO-mediated effect was dependent on ubiquitination, because treatment with MG-132, an inhibitor of proteasomal degradation, prevented receptor loss (Fig. 5b).

Importantly, when we examined ERβ levels in RO-treated breast cancer cells, we found that in a short-term assay, ERβ was increased in both BT-474 and T47-D cells in a time- and dose-dependent manner (Fig. 5c). RO also decreased expression of the survival protein Bcl-2 in breast cancer cells (Fig. 5d). Comparable results for changes in expression of ERα and ERβ in response to RO treatment were obtained in a longer term assay (up to 48 h), using lower concentrations of RO (0.1–10 μM) (Fig. 5e). Thus, our results indicate that treatment of breast cancer cells with RO leads to loss of ERα while simultaneously increasing ERβ. Using BT-474 cells, we found that even lower (nM) doses of RO used for an extended period of time (7 days) degraded ERα and induced

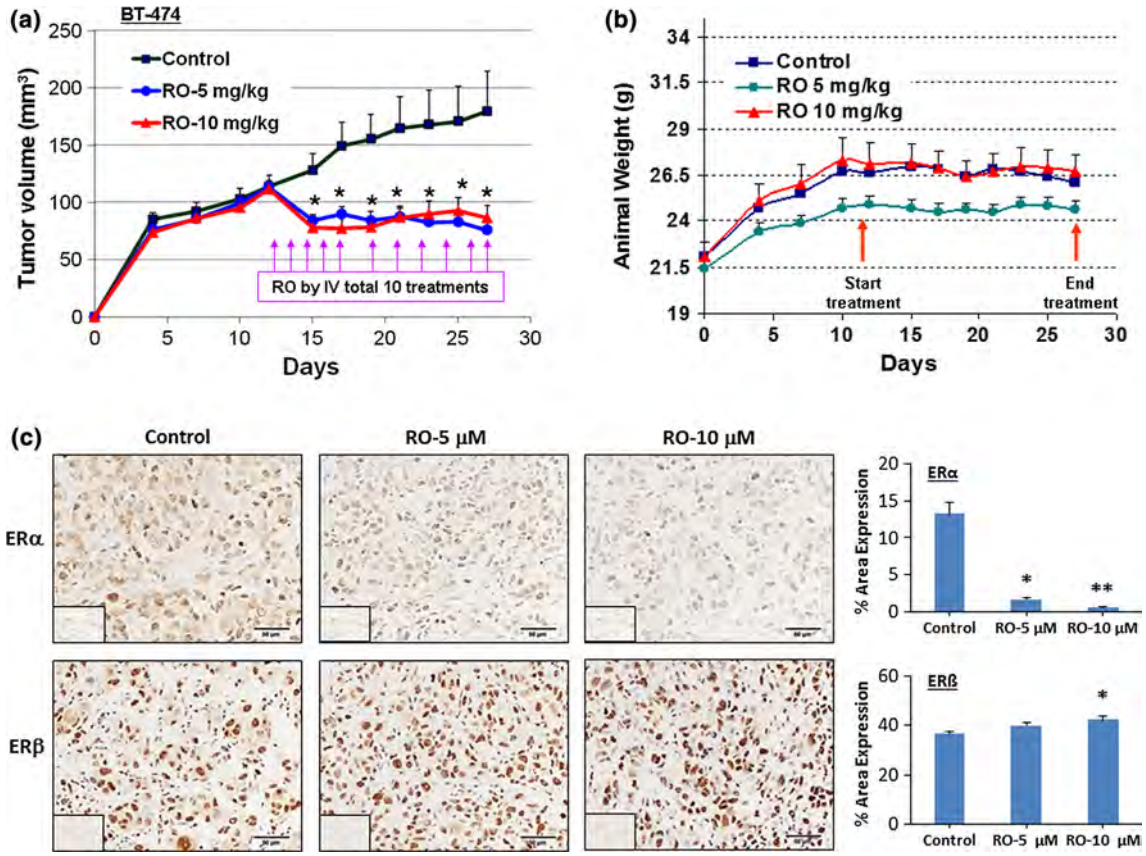

Fig. 4 RO suppresses growth of E-dependent xenografts in nude mice. **a** Six-week-old nude mice received an estradiol slow-release (1.7 mg/60-days release) or placebo pellet by sc implantation 48 h prior to injection with 5×10^6 BT-474 breast cancer cells in Matrigel:DMEM/F12 (4:1; [v/v]) on both flanks. When tumor volumes reached approximately 100 mm^3, animals were treated with RO (5 or 10 mg/kg) or the same volume of PBS (control) daily for 5 days, then every other day for five additional treatments by iv tail-vein injection; mice were given a final RO treatment 2 h before they were sacrificed. Values represent mean ± SEM ($n = 5$). *Significantly different from control ($P < 0.05$ using ANOVA). **b** Animal weight was monitored throughout the experiment. *Arrows* indicate duration of RO treatment. **c** Tumors were collected at end point as shown in **a** and processed for immunohistochemistry and data analysis as described in "Materials and Methods". *Insets* represent negative controls and *bars* represent 50 µm. RO reduced ERα and increased ERβ staining within tumors. *Indicates $P < 0.05$ compared with controls, **denotes significant difference compared with control and treatment with 5 µM RO

ERβ (Fig. 5f, upper panel). In addition to ERβ, p21, an apoptosis and cell-cycle arrest protein, was also induced under these conditions (Fig. 5f, lower panel). p21 was also reduced in T47-D cells (data not shown), suggesting that the effects of RO are not confined to one cell line. Thus, loss of ERα and ERβ induction by RO in breast cancer cells appears to be off-target effects that occur in response to both low and high doses of the OSC inhibitor. Exposure of BT-474 or T47-D breast cancer cells to 25 µM RO did not affect levels of mRNA for either receptor (data not shown), indicating that RO-induced changes in ER levels were independent of RNA transcription.

Finally, we examined the effect of RO analogs, a different class of OSC inhibitor (U18666A), and HMG-CoA reductase inhibitors (statins) on ERα and ERβ levels. When BT-474 cells were exposed to 25 µM RO, three analogs of RO, or U18666A, only the two RO analogs that were found to be most effective in reducing breast cancer cell viability (RO 48-8071 and RO 61-3479; Fig. 1a), caused a loss of ERα and increased ERβ (Fig. 5g). Of the two HMG-CoA reductase inhibitors tested (Simvastatin and Fluvastatin), only Fluvastatin decreased ERα levels. Neither statins elevated ERβ levels (Fig. 5h).

Modulation of ERβ activity modifies the anti-proliferative effects of RO on breast cancer cells

ERβ is known to play an anti-proliferative role in breast cancer cells [25–28, 37]. To determine whether induction of ERβ protein potentiates the anti-proliferative effects of RO, we treated BT-474 cells with RO in the presence

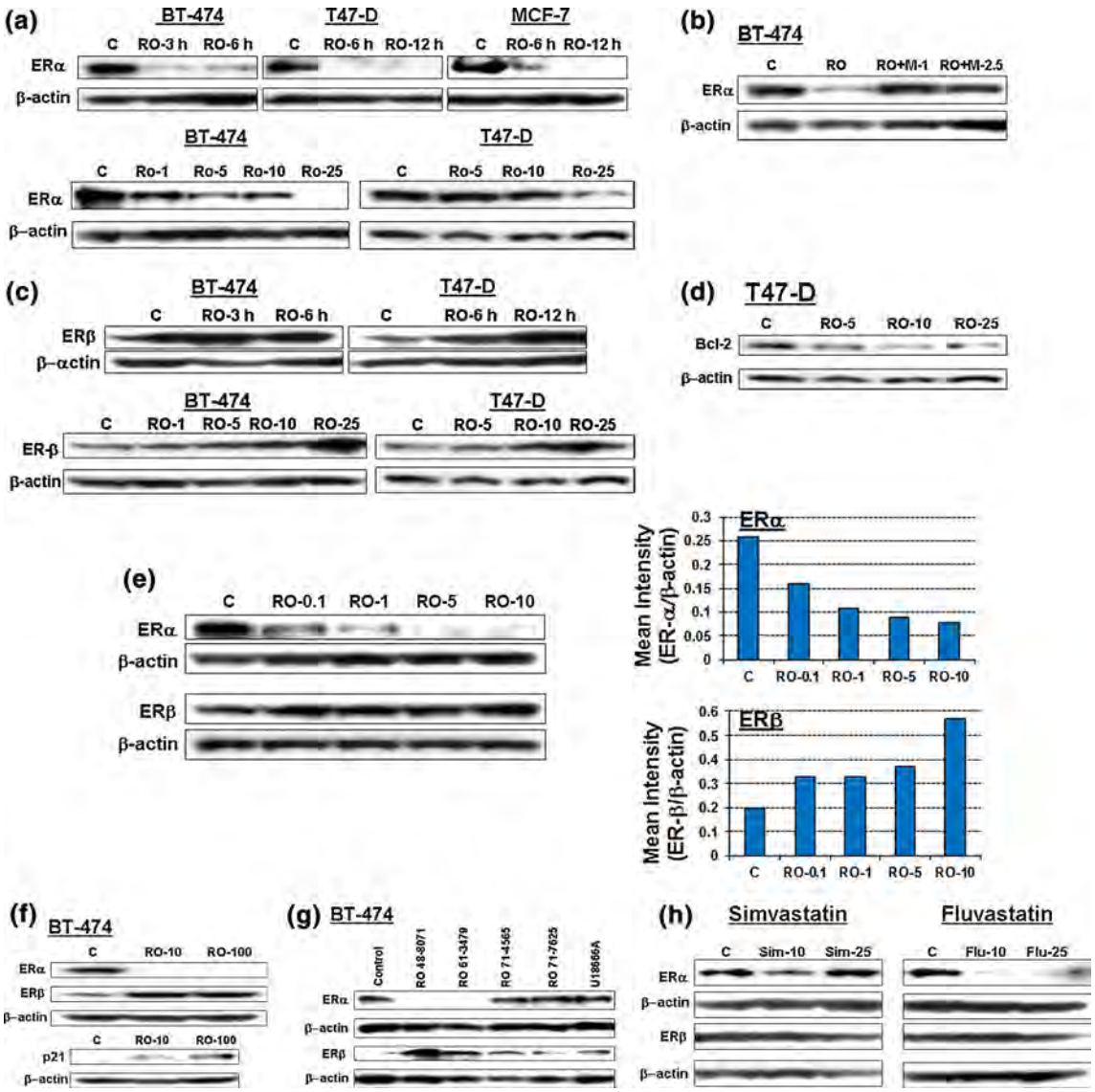

Fig. 5 RO decreases ERα and increases ERβ in breast cancer cells. **a** Breast cancer cells were treated with 0 (control; C), 1, 5, 10, or 25 μM RO for 3 or 6 h (BT-474) or 6 or 12 h (T47-D and MCF-7) in 5 % FBS DMEM:F12. *Upper panel*, all treatments with 25 μM RO; *lower panel*, BT-474 cells were treated for 3 h, and T47-D cells were treated for 6 h. **b** BT-474 cells were treated with 25 μM RO alone or in combination with 1 or 2.5 μM MG-132 (M) in 5 % FBS DMEM:F12 for 3 h. **c** Breast cancer cells were treated with 0 (control; C), 1, 5, 10, or 25 μM RO for 3 or 6 h (BT-474) or 6 or 12 h (T47-D and MCF-7) in 5 % FBS DMEM:F12. *Upper panel*, all treatments with 25 μM RO; *lower panel*, BT-474 cells were treated for 3 h, and T47-D cells were treated for 6 h. **d** T47-D cells were treated with 0 (control; C), 5, 10, or 25 μM RO for 6 h in 5 % FBS DMEM:F12. **e** BT-474 cells were exposed to sub-pharmacological levels of RO (0.1–10 μM) for 48 h. **f** BT-474 cells were treated with 10 or 100 nM RO for 7 days with a media change every 48 h containing fresh RO. **g** BT-474 cells were treated with the indicated compounds at 25 μM for 3 h. **h** BT-474 cells were treated with 10 or 25 μM Simvastatin or Fluvastatin (or vehicle, C) for 3 h. For all panels, whole-cell extracts were subjected to Western blotting to analyze protein expression, and levels of β-actin were assessed as a protein loading control. All experiments were conducted at least twice

of an ERβ agonist DPN. DPN enhanced the effects of RO on reducing the viability of breast cancer cells (Fig. 6a), suggesting that activation of ERβ is partially responsible for RO-mediated effects on breast cancer cell viability. Incubation of BT-474 cells with the ERβ antagonist PHTPP blocked RO-mediated reduction of cell viability (Fig. 6b), providing further evidence that ERβ plays a role in mediating the effects of RO on breast cancer cells. Interestingly, exposure of cells to PHTPP alone also increased cell viability. Similar observations were made with T47-D cells (data not shown).

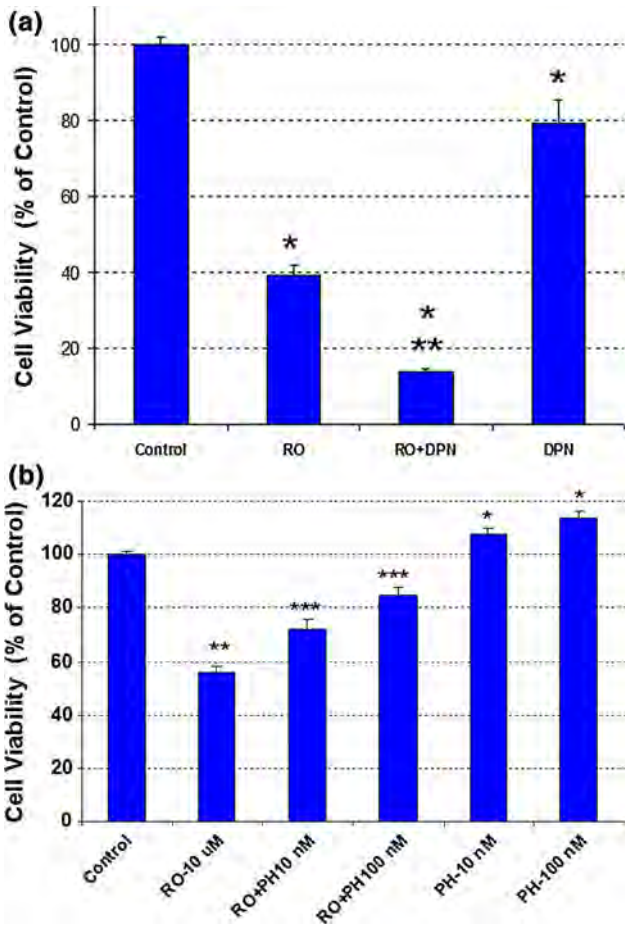

Fig. 6 Modulation of ERβ activity influences RO-mediated effects on breast cancer cell viability. **a** BT-474 cells were treated with 10 μM RO ± 1 μM ERβ agonist DPN or with 1 μM DPN alone (dose taken from ref. [50]) for 48 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control; **significantly different from RO-treatment and DPN-treatment groups ($P < 0.001$, ANOVA). **b** BT-474 cells were treated with 10 μM RO ± 10 nM or 100 nM ERβ antagonist PHTPP (PH) for 24 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly increased compared with control (set at 100 %); **significantly decreased relative to control group; ***significantly different from RO-treatment group

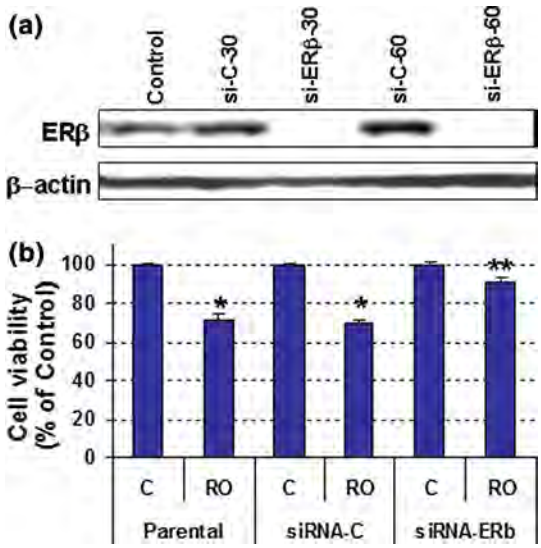

Fig. 7 ERβ knockdown blocks the anti-proliferative effects of RO in breast cancer cells. T47-D cells were transfected with 30 or 60 nM ERβ siRNA (si-ERβ) or scrambled siRNA (si-C) or transfection reagent alone (Control or C) for 72 h. **a** Whole-cell extracts were subjected to Western blotting to analyze ERβ expression. Levels of β-actin were assessed as a protein loading control. **b** Cells transfected with 60 nM siRNA (or T47-D cells transfected with transfection agent alone; parental cells) were treated with RO (10 μM) or vehicle alone (C) for 48 h and cell viability determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from vehicle control group; **significantly different from RO-treated samples in scrambled siRNA group and parental cell group ($P < 0.001$; one-way ANOVA)

Loss of ERβ reduces the anti-proliferative effects of RO in breast cancer cells

Finally, we used siRNA knockdown studies to determine whether RO-induced increases in levels of ERβ were responsible for anti-proliferative effects observed in breast cancer cells. ERβ siRNA but not the scrambled siRNA control effectively knocked down ERβ expression (Fig. 7a). Treatment with RO resulted in a loss of cell viability in both control samples and cells treated with scrambled siRNA. However, when cells were exposed to siRNA specific for ERβ, RO was unable to reduce cell viability to the same extent as it did in control and scrambled siRNA-treated cells (Fig. 7b). These findings support the idea that induction of ERβ is at least partially involved in mediating the effects of RO on reducing cell viability in breast cancer cells.

Discussion

Hormone-dependent breast cancer is the most common type of clinically observed mammary cancer [1, 4, 6]. Although a number of anti-hormonal treatment strategies are currently employed to control progression of the disease, drug-resistant tumors that continue to express ER frequently emerge [1, 4]. As a consequence, studies are ongoing whose goal is to identify new compounds with the ability to control ERα-dependent proliferation in breast tissue and thereby prevent tumor progression. While conducting studies in breast cancer cells to determine the anti-proliferative capacity of cholesterol biosynthesis inhibitors, we discovered that analogs of RO, a class of compound that blocks OSC activity, also down-regulated ERα. Furthermore, RO compounds simultaneously up-regulated the druggable anti-proliferative protein ERβ [25–28, 37], thus

negating the concerns that loss of ERα could lead to hormone-resistant tumors.

We examined the effects of four different RO analogs on ERα-positive breast cancer cell lines. All of them reduced the cell viability, with two particular compounds more potent than the others. We subsequently characterized the effects of RO 48-8071 (RO) as the lead compound on breast cancer cell proliferation and tumor development. RO effectively reduced tumor cell viability in short-term assays (IC_{50} values between 6 and 12 μM; SRB 48-h), while lower concentrations (nM) of RO significantly suppressed the viability of tumor cells in longer term (7 day) assays. We also observed that concentrations of RO up to 10 μM had no effect on the viability of normal mammary cells, suggesting that its in vitro effects are specific to breast cancer cells. Consequently, we propose that since RO appears to be non-toxic to normal cells, it might be used to target tumors with little risk of patient toxicity. Subsequent in vivo studies provide evidence which further supports the use of RO as a therapeutic agent with little or no risk of toxic side effects. Although it is unlikely that RO binds directly to ER due to strict structural requirements for ER-ligand interactions, we will determine whether it binds directly to ERα and ERβ in future studies using competition assays.

In order to compare the effects of RO with the more widely tested HMG-CoA reductase inhibitors (statins), we treated cells with Simvastatin and Fluvastatin, both of which effectively reduced breast cancer cell viability, though with less potency than RO. Our findings, therefore, suggest that RO is more effective than statins at inhibiting breast cancer cell proliferation.

RO suppressed E-induced proliferation of five breast cancer cell lines, including BT-474 cells, which are tamoxifen resistant and which express high levels of HER-2/neu. Tumors that are high in Her2/neu expression have poor prognosis [38, 39]. We administered RO to nude mice bearing BT-474 cell-derived xenografts grown in response to implanted E-containing pellets and observed suppression of tumor growth. This suggests that RO could be an effective means of suppressing cells that are resistant to anti-hormones, though this possibility remains to be tested. Studies are currently underway to determine whether administration of higher levels of RO might promote complete xenograft regression without toxicity.

Since RO suppressed the growth of E-dependent breast cancer cells, we conducted studies aimed at determining whether the OSC inhibitor affects ERα levels. We found that levels of ERα were indeed reduced dramatically in response to RO, in a time- and dose-dependent manner both in vitro and in vivo. BT-474 cells were most sensitive to RO, in accord with their sensitivity in SRB assays. Further in vitro studies showed that the proteasome inhibitor MG-132 completely blocked receptor loss, indicating that RO induces proteasome-mediated receptor degradation. Ubiquitination has previously been shown to control ER degradation [40]. ERα mRNA synthesis was not reduced by RO treatment, suggesting that the loss of ERα is a post-transcriptional event. The term selective ER down-regulator or degrader has been used to describe the effect of therapeutic agents that degrade ERα, and RO seems to be another member of this class [41, 42]. Previously, the anti-estrogen ICI-182,780 has been shown to cause a similar loss of ERα in breast cancer cells [41]; however, its use in long-term treatment is restricted due to bone-related toxicities in post-menopausal women [43]. Further studies are needed with RO to determine its effect on bone after a long-term use.

Importantly, we found that as well as degrading ERα, pharmacological concentrations of RO concomitantly increased ERβ levels in vitro. However, while induction of ERβ was significant in vitro, its up-regulation was not as robust in vivo. This was most likely due to tumors being collected at the end point, several days after the initial treatment of nude mice bearing BT-474 xenografts. It is, therefore, likely that we missed the higher levels of ERβ, which were subsequently lost when cells expressing elevated levels of ERβ underwent apoptosis. However, this requires confirmation by collection of tumors a few days after initial treatment with RO and assessment of ERβ induction. The consistent in vitro induction of ERβ in various cell lines was most likely due to short-term exposure to the drug. In any event, the loss of ERα over time leads to a high ratio of ERβ to ERα, a scenario which has been shown to inhibit tumor cell proliferation [44]. We further characterized the in vitro effect of RO by real-time PCR analysis and found that the OSC inhibitor did not affect levels of ERβ mRNA. Thus, it would appear that RO likely stabilizes ERβ protein over time. In future studies, we will examine in more detail just how RO influences ERβ protein stability. Because BT-474 cells were most sensitive to the anti-proliferative effects of RO, we exposed this cell line to lower levels of RO and assessed ERβ induction. Doses of RO as low as 100 nM induced ERβ, while simultaneously degrading ERα, while in a 7-day assay, 10 nM RO completely eliminated ERα, while also inducing ERβ (Fig. 5f).

Of the several RO analogs tested, the two that reduced breast cancer cell viability most effectively also potently reduced ERα levels while at the same time inducing ERβ. Those that did not degrade ERα still induced ERβ to some extent. Based on these findings, we conclude that increased ERβ is the predominant off-target factor that accounts for loss of breast cancer cell viability following exposure to analogs of RO at least in vitro. Interestingly, HMG-CoA reductase inhibitors demonstrated a variable response;

while Simvastatin did not degrade ERα to the same degree as the two most effective RO analogs, Fluvastatin exerted a comparable effect in this regard. Neither Simvastatin nor Fluvastatin induced ERβ. It is clear that the two classes of cholesterol biosynthesis inhibitor exert differential effects on the ratio of ERα and ERβ in breast cancer cells. This ratio has been shown to be an important predictor of cell growth; a high ratio of ERα/ERβ is proliferative, whereas increased expression of ERβ is associated with loss of tumor cell proliferation [45–48].

To further confirm that ERβ is at least partially responsible for loss of breast cancer cell viability, we blocked receptor activity using PHTPP, a selective ERβ antagonist. PHTPP suppressed the anti-proliferative activity of RO in a dose-dependent manner. We further confirmed the important role played by ERβ in reducing cell viability by exposing breast cancer cells to DPN, an ERβ-specific agonist. When administered individually, both RO and DPN inhibited breast cancer cell viability. However, when a combination of the two compounds was given, their inhibitory effect was additive, an outcome that may be due to increased cellular levels of ERβ in response to RO. Down-regulation of ERβ by siRNA significantly reduced the anti-proliferative effects of RO, providing further evidence of the importance of ERβ in mediating RO effects on breast cancer cell viability. It therefore appears likely that drugs that increase ERβ activity in breast cancer cells could be made even more effective when administered in conjunction with RO. The development of therapeutic regimens using a combination of two agents might make it possible to manage disease using lower levels of both, reducing the likelihood of toxic side effects that result from current therapeutic modalities [49].

In summary, the data presented in this manuscript strongly suggest that, in addition to its ability to suppress cholesterol biosynthesis, the OSC inhibitor RO exerts a powerful anti-tumor effect by the off-target loss of ERα and induction of the anti-proliferative protein ERβ. The loss of ERα but not ERβ in vivo leads to a large increase in the ERβ/ERα ratio, which could be responsible for tumor loss [43–48]. In addition, in vitro data show that ERβ can promote some of the anti-tumor properties of RO. Thus, we propose that ERβ is at least partially responsible for the observed suppression of breast cancer cell viability and suggest, therefore, that combination therapy using inhibitors of cholesterol biosynthesis (such as RO) together with commonly used chemotherapeutic drugs could prove beneficial as a means by which to suppress breast cancer progression. We are currently conducting studies to determine the effectiveness of such combination therapies.

Acknowledgments Supported by a Department of Defense Breast Cancer Program Grant W81XWH-12-1-0191, the National Institutes of Health Grant R21 GM088517, and by a Faculty Research Grant from the University of Missouri, Columbia. SMH is the Zalk Missouri Professor of Tumor Angiogenesis. Funds to purchase the nanodrop instrument were provided through the generosity of numerous donors to the Ellis Fischel Cancer Center.

Conflict of interest JDA is an employee of F. Hoffmann-La Roche AG. All other authors declare that they have no conflict of interest.

Ethical standards The experiments comply with the current laws of the country in which they were performed (USA).

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Cholesterol synthesis inhibitor RO 48-8071 suppresses transcriptional activity of human estrogen and androgen receptor

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Received May 6, 2014; Accepted June 18, 2014

DOI: 10.3892/or.2014.3332

Abstract. Breast cancer cells express enzymes that convert cholesterol, the synthetic precursor of steroid hormones, into estrogens and androgens, which then drive breast cancer cell proliferation. In the present study, we sought to determine whether oxidosqualene cyclase (OSC), an enzyme in the cholesterol biosynthetic pathway, may be targeted to suppress progression of breast cancer cells. In previous studies, we showed that the OSC inhibitor RO 48-8071 (RO) may be a ligand which could potentially be used to control the progression of estrogen receptor-α (ERα)-positive breast cancer cells. Herein, we showed, by real-time PCR analysis of mRNA from human breast cancer biopsies, no significant differences in OSC expression at various stages of disease, or between tumor and normal mammary cells. Since the growth of hormone-responsive tumors is ERα-dependent, we conducted experiments to determine whether RO affects ERα. Using mammalian cells engineered to express human ERα or ERβ protein, together with an ER-responsive luciferase promoter, we found that RO dose-dependently inhibited 17β-estradiol (E2)-induced ERα responsive luciferase activity (IC_{50} value, ~10 μM), under conditions that were non-toxic to the cells. RO was less effective against ERβ-induced luciferase activity. Androgen receptor (AR) mediated transcriptional activity was also reduced by RO. Notably, while ERα activity was reduced by atorvastatin, the HMG-CoA reductase inhibitor did not influence AR activity, showing that RO possesses broader anti-tumor properties. Treatment of human BT-474 breast cancer cells with RO reduced levels of estrogen-induced PR protein, confirming that RO blocks ERα activity in tumor cells. Our findings demonstrate that an important means by which RO suppresses hormone-dependent growth of breast cancer cells is through its ability to arrest the biological activity of ERα. This warrants further investigation of RO as a potential therapeutic agent for use against hormone-dependent breast cancers.

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Key words: cholesterol inhibitor, oxidosqualene cyclase inhibitor, RO 48-8071, estrogen receptor-α, estrogen receptor-β, androgen receptor

Introduction

It is becoming increasingly apparent that cholesterol and its metabolites contribute to the development of breast cancer (1-3). Various mechanisms by which cholesterol promotes the growth of breast tumors have been identified; cholesterol is also the precursor of steroid hormones such as estrogen and testosterone, both of which have well-recognized tumor promoting effects (4). The cholesterol metabolite 27-hydroxycholesterol has been shown to exert selective estrogen receptor modulation (SERM) effects (5,6) and also to promote the growth of estrogen receptor (ER)-positive breast tumors (3). Current evidence, therefore, suggests that by disrupting cholesterol biosynthesis, we may inhibit cell cycle progression and induce cell death (7). Most previous studies have targeted HMG-CoA reductase, a rate limiting enzyme in the cholesterol biosynthetic pathway. However, using *in silico* analysis, we recently found that oxidosqualene cyclase (OSC), which is downstream of HMG-CoA reductase and is a critical enzyme that catalyzes the cyclization of 2,3-oxidosqualene to lanosterol, may also be a potential target by which to control the proliferation of ER+ve tumors (8). On the basis of these initial findings, and after examining the effects of different OSC inhibitors on human breast cancer cells, we selected RO 48-8071 (RO) as a prototype inhibitor. Our decision to use RO was also based on the aforementioned study where we showed that the compound is a suitable candidate for suppressing human breast cancer cells *in vitro* (8).

Since there are differences in intra-tumor estrogen levels between ER+ve and triple negative breast cancer tissue (9), we hypothesized that the levels of enzymes involved in the cholesterol biosynthetic pathway may be altered in breast cancer cells. This could include differential expression or activity of OSC. Taking this into consideration, we measured levels of OSC in a panel of human breast cancers and normal tissues and found no significant difference in levels of OSC

mRNA between steroid responsive and hormone-independent tumors. Furthermore, OSC expression in tumor tissue was not significantly different than in normal mammary tissue, suggesting that RO must suppress breast cancer cell growth via alternative, off-target effects. In this respect, we discovered that RO suppresses the transcriptional activity of ERα and to some extent that of ERβ, under conditions that preserve cell viability. Moreover, RO also suppresses androgen receptor (AR) transcriptional activity, another major determinant of breast cancer progression (10). Using western blot analysis, we verified that RO suppresses levels of progesterone receptor (PR), the expression of which is directly controlled by ERα in human breast cancer cells (11). This confirms that the ERα-mediated signal transduction pathway is inhibited when cells are exposed to RO.

Materials and methods

RO 48-8071 was purchased from Sigma, dissolved in DMSO and stored in aliquots at -20˚C prior to use.

Human tissue qPCR. qPCR-ready TissueScan™ cDNA Array (OriGene starter kit, cat. no. TSRT101) was initially used to determine the expression of OSC in human breast cancer tissues following guidelines recommended by the manufacturer. Human breast cancer tissue array (cat. no. BCRT101) was then used to measure OSC expression in tissues that either expressed ER, PR and HER2 (hormone-dependent) or tissues lacking these receptors (triple negative) at different stages of development (stage I-III). Human OSC (Hs00158906_ml LSS) TaqMan Fam probes were obtained from Applied Biosystems and normalized with human GAPDH (Hs03929097_g1 FG). Relative gene expression was determined using the following formula: Fold-change in gene expression, $2\text{-}\Delta\Delta Ct = 2 - \{\Delta Ct$ (cancer tissue samples) - ΔCt (normal tissue)$\}$, where $\Delta Ct = Ct$ (OSC) - Ct (GAPDH), where Ct represents threshold cycle number.

Luciferase activity. Receptor assay systems were obtained from Indigo Biosciences (State College, PA, USA) and luciferase activity was determined following the manufacturer's recommended protocol. The assays comprised non-human mammalian cells engineered to express human ERα, ERβ, androgen receptor (AR) protein and luciferase reporter gene functionally linked to the corresponding nuclear receptor -responsive promoter. We initially used Indigo Biosciences Receptor Assay (cat. no. IB00421-48P) to determine the effects of RO on estradiol-induced ERα and ERβ activity. ICI 182,780 was used as a reference antagonist to 17β-estradiol. Since our initial results showed more pronounced effects of RO on ERα activity compared with ERβ, we focused on ERα-promoter linked activity using Indigo Biosciences Receptor Assay (cat. no. IB00401). We also determined the effects of a commonly used cholesterol inhibitor, atorvastatin (Ator) on ERα promoter-linked activity. Since there is increasing evidence to support the role of AR in the development of breast cancer (10), we conducted studies to assess the effects of RO on AR-dependent luciferase activity. A human AR reporter assay system (cat. no. IB03001; Indigo Biosciences) was used with 6α-F1 testosterone as the reference agonist for AR. For all reporter studies, we sequentially used fluorescence-based LCM assays (cat. no. LCM-01; Indigo Biosciences), following the manufacturer's guidelines, to determine the relative number of live cells at the assay endpoint. All luciferase assays were read with GloMax®-Multi+ Microplate Multimode Reader system (Promega).

Western blotting. BT-474 breast cancer cells were grown in a humidified atmosphere of 5% CO_2 at 37˚C in 100-mm cell culture plates using phenol red-free Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum. When cells reached 50-60% confluence, they were washed with PBS and switched into DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC) for 24 h. Cells were then washed, transferred into fresh DMEM/F12 and treated with E2 (10 nM) in the absence and presence of RO (5 µM) or with RO alone. Cells were treated with RO for 3 h prior to treatment with E2 for 16 h, after which cells were harvested. Nuclear protein was extracted following the manufacturer's guidelines (cat. no. 40010; Active Motif, USA). Protein aliquots (20 µg) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and blotted with the following human specific antibodies: ERα (SC-8005, 1:200 dilution), ERβ (SC-8974, 1:200 dilution), PR (SC-810, 1:200 dilution) (all from Santa Cruz Biotechnology), β-actin (Sigma-Aldrich, St. Louis, MO, USA). Protein bands were detected and quantified by blotting with anti-mouse secondary antibody (SC-2005, 1:10,000 dilution), or anti-rabbit secondary antibody (SC-2004, 1:10,000 dilution) (both from Santa Cruz Biotechnology), and a chemiluminescent detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Real-time PCR. Total RNA was extracted from two distinct breast cancer cell lines, BT-474 and T47D cells, which express both ERα and ERβ. Cultured cells were treated with 20 µM RO for 1, 3, 6 and 24 h, respectively. Control samples were treated with ethanol, the vehicle medium in which RO was dissolved. RNA was extracted using an EZ-Bioresearch RNA Isolation kit (cat. no. R1002-50) according to the manufacturer's instructions. Two micrograms of RNA were reverse transcribed into cDNA using a high capacity DNA synthesis kit (cat. no. 4368814; Applied Biosystems). cDNA was then amplified using an ABI 7300 Real-Time PCR Instrument (Applied Biosystems), specific TaqMan primers and TaqMan® Universal PCR Master Mix. Human ERα (Hs00174860_m1), ER-β (Hs00230957_m1) and GAPDH (Hs03929097_g1 FG) TaqMan Fam probes were used (Applied Biosystems). Relative gene expression was determined using the following formula: Fold-change in gene expression, $2\text{-}\Delta\Delta Ct = 2 - \{\Delta Ct$ (treated samples) - ΔCt (untreated control samples)$\}$, where $\Delta Ct = Ct$ (ERα or ERβ) - Ct (GAPDH), where Ct represents threshold cycle number. All reactions were carried out in duplicate for at least three independent experiments.

Statistical analysis. Comparisons were made between multiple groups by analysis of variance (ANOVA) with Neuman-Keuls post-hoc testing. Where normality was not achieved, non-parametric ANOVA (Kruskal-Wallis) and post-hoc Dunn's test was performed. Significance was defined as $p<0.05$. Unless indicated, values are shown as mean ± SEM.

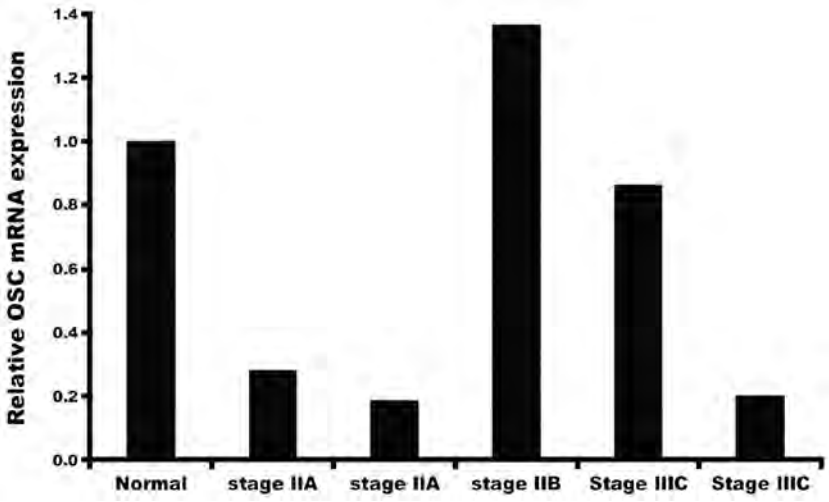

Figure 1. Relative OSC mRNA expression in breast cancer at different stages of growth. Human qPCR-ready TissueScan™ cDNA Arrays (cat. no. TSRT101) were obtained from OriGene and real-time PCR was conducted as described in Materials and methods. Results showed variable levels of expression of OSC mRNA within different tumors compared with corresponding normal tissue. OSC, oxidosqualene cyclase.

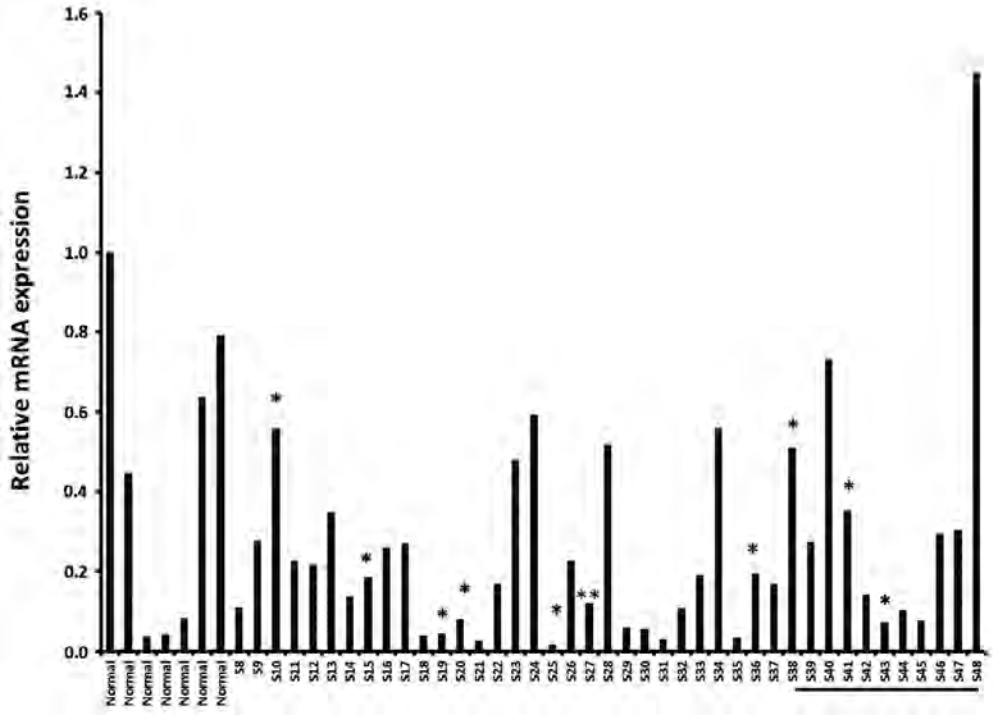

Figure 2. Relative OSC mRNA expression in distinct breast cancer tissues at different stages of growth (stage I-III). Human qPCR-ready TissueScan™ cDNA Arrays (cat. no. BCRT101) were obtained from OriGene and real-time PCR was conducted as described in Materials and methods. Results showed no significant difference in OSC mRNA expression between hormone-dependent (ER^+, PR^+, $HER2^+$) and triple negative breast cancer at various stages of growth. *Triple negative breast tumor, **ER/PR-negative/Her-2neu high tumors; remaining bars represent ER/PR positive tumors. OSC, oxidosqualene cyclase.

Results

OSC mRNA expression in human cancer tissues. In order to measure OSC expression we employed ready to use qRT-PCR human tissue cDNA arrays to obtain preliminary data on levels of OSC mRNA in a limited number of tumors collected at various stages of development (Fig. 1). Our initial study showed that varying levels of OSC message are present in samples of tumor obtained at different stages, and that, depending on the sample, mRNA levels may be higher or lower than levels present in normal breast tissue. This prompted us to analyze a larger cohort of samples and to focus on different breast cancer tissues at different stages (stage I-III) available from OriGene as described in Materials and methods. As shown in Fig. 2, there was no consistency in levels of OSC expression, either within tissue from normal breast, or within breast tumor samples collected at various stages, including triple negative cancers.

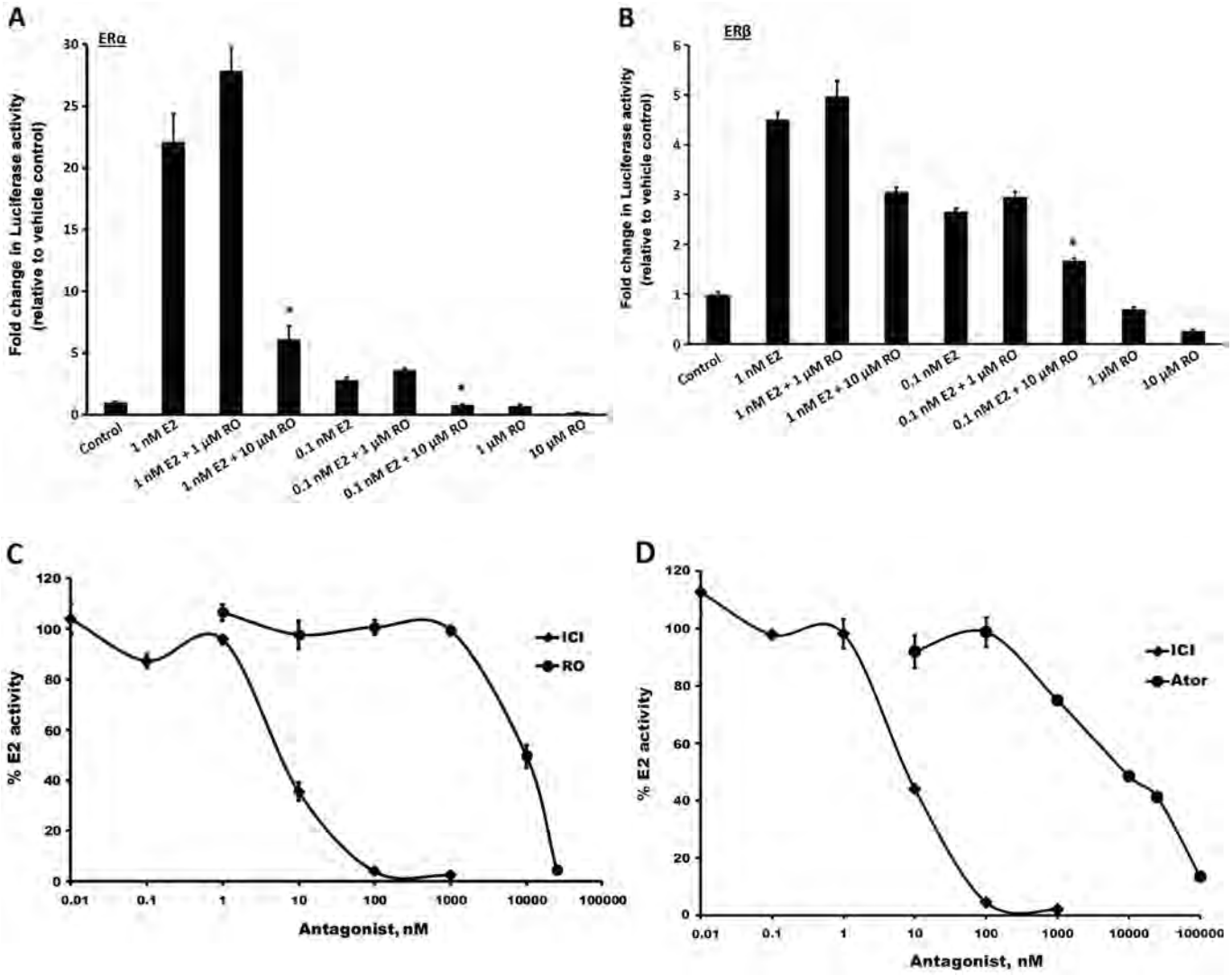

Figure 3. RO significantly inhibits 17β-estradiol induced ER-mediated luciferase activity. Non-human mammalian cells were engineered with human (A) ERα and (B) ERβ expression plasmid and luciferase reporter gene functionally linked to estrogen response element. Cells were seeded in 96-well plates according to the manufacturer's protocol. Cells were treated with 0.1 or 1 nM 17β-estradiol (E2) ±1 or 10 μM RO for 18 h, after which luciferase activity was determined. Results showed that 10 μM RO significantly inhibited E2-induced luciferase activity with both ERα and ERβ. * $p<0.05$, significantly different from group treated with similar concentration of E2. (C) RO dose-dependent inhibition of ERα-mediated luciferase activity. Cells were seeded in 96-well plates as described above and treated with 1 nM E2 alone or in combination with either ICI 182,780 (ICI) at concentrations varying from 0.01-1,000 nM or RO at concentrations between 1.0 and 50,000 nM. Results are expressed as percentage of maximum luciferase activity obtained with 1 nM E2. (D) Atorvastatin (Ator) dose-dependently inhibited ERα-linked luciferase activity. Cells were seeded in 96-well plates as described above and treated with 1 nM E2 alone or in combination with either ICI at concentrations varying from 0.01-1,000 nM or Ator at concentrations between 1.0-100,000 nM. Results are expressed as percentage of 1 nM E2 activity. RO, RO 48-8071.

Effects of RO on ERα, ERβ and AR-promoter linked hormone responsive luciferase activity. In a previous study, it was indicated that RO reduces breast cancer cell viability in a dose-responsive manner (8). However, since we found levels of OSC mRNA in breast tumors to be inconsistent (Figs. 1 and 2), we concluded that it was unlikely that RO exerts its effects on breast cancer cells primarily by targeting OSC. Previously, we showed that pharmacological levels of RO degrade ERα in breast cancer cells (Liang *et al* unpublished data). In the present study, we examined whether lower levels of the drug also influenced the transcriptional activity of ER, without degrading the receptor. For these studies, we used steroid receptor-driven luciferase reporter assays (Indigo Biosciences) and concentrations of RO that did not affect cell viability, as determined from a previous study (8), or ERα protein levels. We found that treatment with 1 nM 17β-estradiol for 22 h increased luciferase activity in both ERα and ERβ-linked reporter cells (Fig. 3A and B; bars on left). Induction was much stronger with ERα than ERβ, an observation in accordance with a previous study (12). RO caused a decrease in E2-mediated transcriptional response in a manner that was dose-dependent. Both ERα and ERβ luciferase activities were reduced in response to RO (Fig. 3A and B), although RO-mediated transcriptional suppression was much greater with 10 μM RO when ERα activity was assessed (>50%) than when ERβ induced transcription was measured (<50%).

Having examined the effects of RO on transcriptional activity mediated by both ERα and ERβ, we used ICI 182,780, a well-characterized ERα antagonist, to specifically suppress ERα-mediated transcription (Fig. 3C). ICI 182,780 inhibited

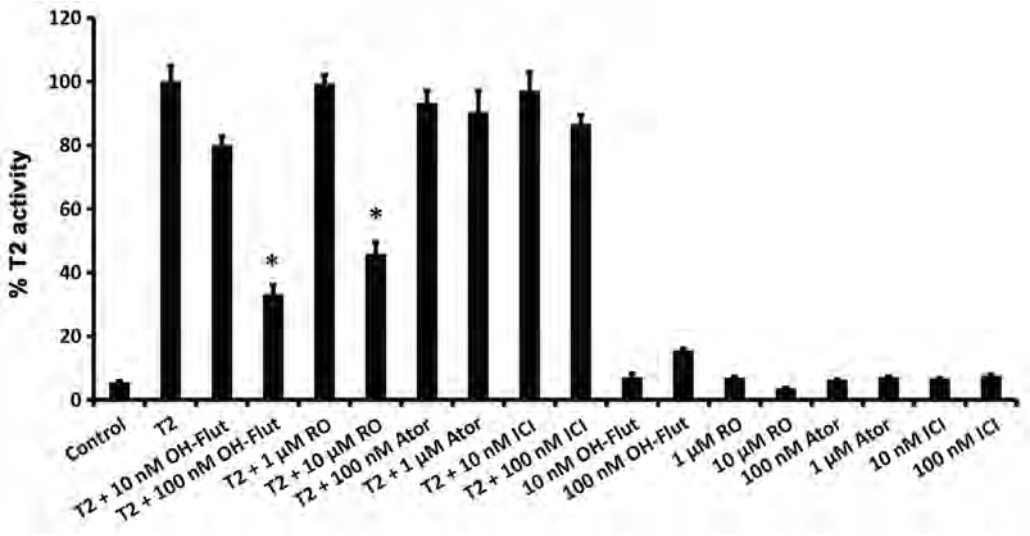

Figure 4. RO significantly inhibits 6α-testosterone-induced AR linked luciferase activity. Non-human mammalian cells were engineered to express human AR protein and luciferase reporter gene functionally linked to corresponding AR-responsive luciferase promoter. Cells were seeded in 96-well plates and treated with 400 pM 6α-testosterone (T2) alone or in combination with one of the following at the concentrations given below; RO (1 or 10 μM), ICI (10 or 100 nM), Ator (100 nM or 1 μM) or flutamide (OH-flut) (10 or 100 nM), or the various compounds alone. After 18 h, luciferase activity was determined following the manufacturer's guidelines. Results showed that both RO and OH-flut significantly inhibited T2 induced AR-linked luciferase activity in a dose-dependent manner while ICI and Ator did not affect luciferase activity. * $p<0.05$, significantly different from T2 group. RO, RO 48-8071; AR, androgen receptor; Ator, atorvastatin.

transcriptional activity considerably more potently than RO (~1,000-fold), although, notably, atorvastatin, another cholesterol biosynthesis inhibitor that acts on HMG-CoA reductase and is commonly used in humans to lower cholesterol levels, inhibited ERα-mediated transcriptional activity at a level comparable to RO (Fig. 3D).

Markedly, RO also inhibited a 6α-testosterone-mediated increase in AR transcriptional activity, which was also blocked by the AR antagonist hydroflutamide (OH-flut; Fig. 4). However, in contrast to their suppression of ERα-mediated transcriptional activity (Fig. 3), neither atorvastatin nor ICI 182,780 had any inhibitory effect on AR-mediated transcription (Fig. 4).

In order to ensure that the observed reduction in luciferase reporter activity was not due to induced cell apoptosis, a fluorescence-based LCM assay kit (LCM-01; Indigo Biosciences) was used concurrently to measure the proportion of live cells in wells where luciferase activity was measured. As shown in Fig. 5, while the proportion of live cells was significantly reduced by the apoptosis-inducing compound staurosporine, all the other compounds tested in the present study were non-toxic at the concentrations used.

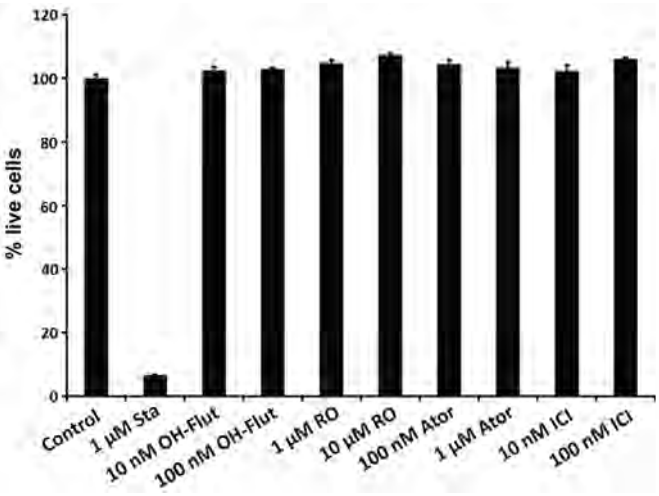

Figure 5. RO and other compounds tested in the present study do not show cytotoxic effects at the concentrations used. A fluorescence-based LCM assay kit (LCM-01; Indigo Biosciences) was used concurrently to assess the proportion of live cells in wells where luciferase activity was measured. Staurosporine (4 μM) was used as a positive control for apoptosis and cell loss. RO, RO 48-8071.

Effect of RO on estradiol-induced PR expression in BT-474 human breast cancer cells. Confirmation that RO was able to modify the biological activity of ERα was obtained in BT-474 cells in which ERα regulates PR levels by increasing PR gene transcription (11). As shown in Fig. 6A, treatment of BT-474 cells with 10 nM E2 for 16 h resulted in increased levels of both PRA and PRB. E2-mediated increases in PR levels were blocked by RO (5 μM), without any significant loss of ERα. Real-time PCR confirmed that 5 μM RO had no effect on ERα mRNA expression in human BT-474 or T47-D breast cancer cells (Fig. 6B).

Discussion

There is growing evidence to suggest that cholesterol and its metabolites play an important role in the development of breast cancer (1-3). Previous studies showed that inhibition of cholesterol synthesis at different enzymatic steps of the biosynthetic pathway leads to a suppression of cell growth (13,14), although the significance of OSC in tumor progression remains unclear. OSC is a critical enzyme which is involved in the cyclization of 2,3-oxidosqualene to lanosterol and an increase in its

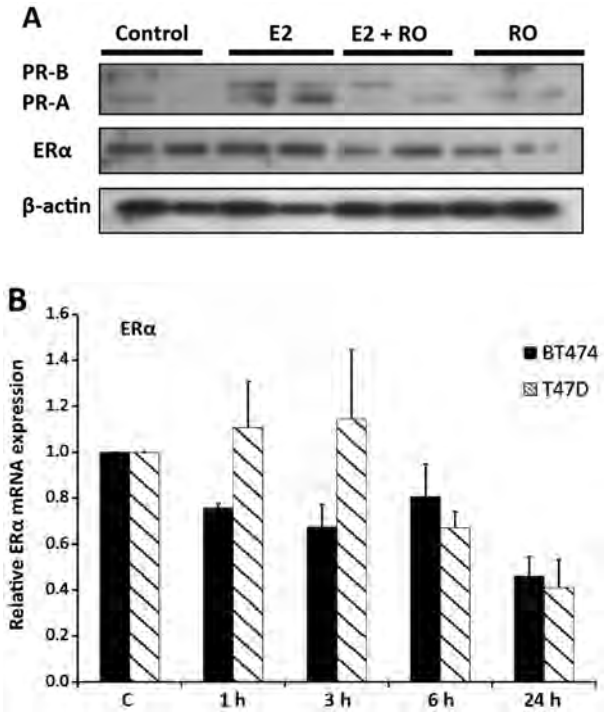

Figure 6. (A) RO inhibits 17β-estradiol-induced PR protein expression in BT-474 human breast cancer cells. BT-474 cells were treated with either DMSO (vehicle medium) or 5 μM RO for 3 h prior to treatment with 10 nM 17β-estradiol for 16 h. Protein was then extracted and western blot analysis performed as described in Materials and methods. Results showed that RO reduced E2-induced levels of PR protein. (B) Relative ERα mRNA expression in human BT-474 and T47-D breast cancer cells treated with RO as described in Materials and methods. Results showed no significant changes in ERα mRNA expression. RO, RO 48-8071.

intra-tumor expression or activity has the potential to raise the levels of cholesterol metabolites with estrogenic activities. Such an increase could result in subsequent proliferative effects within breast cancer cells. In earlier studies involving *in silico* analysis, we showed that RO, an inhibitor of cholesterol synthesis that targets OSC, is a potent ligand with chemotherapeutic properties, which reduces breast cancer cell viability (8). This led us to investigate whether RO may have potential as a chemotherapeutic agent against a broader range of breast cancer cells.

Our initial goal in the present study was to determine whether hormone-responsive and hormone-independent tumors express OSC differently. We hypothesized that differences in OSC expression may partially account for both increased cholesterol biosynthesis and higher levels of intracellular estrogen, which could promote tumor growth. After extensive analysis of a variety of tumor tissues collected at different stages of development and from varying types of tumor, we concluded that there were no significant differences in levels of OSC expression between normal, hormone responsive, hormone-independent and triple-negative breast cancers. It remains to be established, however, whether OSC protein levels are different between the various types of tumor. Based on these observations, we concluded that expression of OSC is unlikely to be a prognostic marker for breast cancer.

In our previous study, we showed that RO significantly reduced the viability of ERα-positive breast cancer cells (8, Liang *et al* submitted). However, since levels of OSC expression did not vary between different tumor types, we concluded that it is unlikely that the ability of RO to disrupt tumor cell proliferation is due entirely to its inhibition of OSC. Since ERα is a major determinant for human breast cancer cell proliferation, we conducted studies to determine whether RO targets ERα, initially using non-human mammalian cells engineered to express human ERα protein, and luciferase reporter gene functionally linked to an ERα-responsive promoter to assess 17β-estradiol induced luciferase activity. Changes in luciferase expression in cells treated with RO and other test compounds provided a sensitive surrogate measure of changes in ERα transcriptional activity without cellular toxicity. As expected, ICI 182,780, a well characterized antagonist which acts partially through downregulation of the receptor (15), blocked the effects of 17β-estradiol on ERα. RO also inhibited 17β-induced ERα-linked luciferase expression in a dose-dependent manner, although higher doses of RO were required to achieve levels of inhibition comparable to ICI 182,780 (Figs. 3 and 4). RO also blocked ERβ activity, although less potently than its inhibition of ERα. Notably, atorvastatin, an alternative inhibitor of cholesterol biosynthesis which inhibits HMG-CoA reductase, also blocked ERα-mediated transcriptional activity. To determine whether RO, atorvastatin and ICI 182,780 exert differential effects on other steroid receptors, we examined their capacity to inhibit transcriptional activity of AR, our rationale being that AR is also involved in breast cancer progression (10). Among the three ligands tested (RO, atorvastatin and ICI 182,780) only RO inhibited AR-mediated transcription, suggesting that the OSC inhibitor possesses even broader effects than we first anticipated. The ability to block both ER and AR effects possibly indicates that, compared with other chemotherapeutic drugs, RO may have additional advantages with respect to its properties as an anti breast cancer agent.

In our studies using Indigo kits to measure transcriptional activity of nuclear receptors, we utilized non-mammalian cells transfected with human receptor. Following these analyses, we confirmed that RO also affects human breast cancer cells by determining its ability to modify the transcriptional activity of ERα in human BT-474 cells. This was achieved by assessing the effects of 17β-estradiol on ERα-mediated induction of PR protein, measuring levels of the latter by western blotting. PR is directly regulated by ERα in BT-474 breast cancer cells in a ligand-dependent manner (11). Using western blot analysis, we found that RO suppressed induction of PR by 17β-estradiol in human BT-474 breast cancer cells (Fig. 6A). Concomitant analysis of ERα mRNA levels showed that under these conditions, RO did not affect expression of ERα. This confirms, in human breast cancer cells, that RO has the ability to suppress the biological functions of ERα without reducing its levels (Fig. 6A and B).

Given that ERα-induced signal transduction controls the growth of the majority of breast cancers (16), the results reported in the present study suggest that RO has the potential to be an effective chemotherapeutic agent against hormone-responsive breast cancer. Based on our observations of its ability to inhibit the biological activities of both ER and AR, further in-depth analysis of the effects of RO on human breast cancer cells is warranted.

Acknowledgements

The present study was supported by a Department of Defense Breast Cancer Program grant no. W81XWH-12-1-0191, and by a Faculty Research Grant from the University of Missouri, Columbia. S.M.H. is the Zalk Missouri Professor of Tumor Angiogenesis. Funds to purchase the nanodrop instrument were provided through the generosity of numerous donors to the Ellis Fischel Cancer Center. The authors thank Mr. Jason Lee for his assistance with the figures.

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Poster Presentation

Liang, Y., Zou, X., Besch-Williford, C., Aebi, JA. and **Hyder , S. M.** (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. Yayun Liang[1,2], Xiaoqin Zou[1,3] , Cynthia Besch-Williford[4], Johannes Aebi[5] and Salman M Hyder[1,2]. Dalton Cardiovascular Research Center[1] & Dept of Biomedical Sciences[2], Department of Physics and Astronomy, Department of Biochemistry, and Informatics Institute[3], University of Missouri, Columbia, MO 65211, IDDEX RADIL[4], Columbia, MO, 65201, and F. Hoffmann-La Roche Ltd., Pharmaceutical Division, CH-4070 Basel, Switzerland[5].

Most human breast cancers are hormone responsive, depending on estrogens and progestins for tumor cell proliferation. Initially, hormone-responsive tumors respond to endocrine therapy, however, most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. It is well-established that breast cancer cells have the capacity to synthesize endogenous cholesterol, the precursor for steroid hormones. Cholesterol biosynthesis by tumor cells therefore potentially contributes towards anti-hormone resistance. Most commonly used cholesterol lowering drugs inhibit HMG CoA-reductase, a key rate-limiting enzyme in the cholesterol biosynthetic pathway; these inhibitors are however associated with certain undesirable side effects that limit their use for cancer therapy. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase, effectively arrested breast cancer cell proliferation. RO0488071 [4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]) (RO), and an analogue RO0613479, were particularly effective in this regard. Administration of both of these OSC inhibitors to ER positive human breast cancer cells (e.g. BT-474, T47-D, MCF-7) at a pharmacological dose or at a dose close to the IC_{50} value for OSC (nM range) reduced tumor cell viability in vitro. Administration of RO to animals with human breast cancer cell-derived xenografts prevented further in vivo progression of the disease, with no apparent toxicity. Since BT-474 cells are also tamoxifen resistant and rich in Her2/neu, RO appears to be effective in vivo against even the most aggressive anti-hormone resistant tumors. Importantly, RO had no effect on the viability of normal human mammary cells. Our study shows for the first time that inhibition of cholesterol biosynthesis using OSC inhibitors is a novel and potent means by which to destroy human breast cancer cells, though further studies are necessary to determine the mechanism of RO mediated loss of breast cancer cell viability. Supported by a Dept of Defense Breast Cancer Pgm grant W81XWH-12-1-0191, NIH grant R21 GM088517, and by a COR grant from the University of Missouri, Columbia.

Poster Presentation

Mafuvadze, B., Liang, Y., Hyder, S. M. (2014) Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells. 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo, Chicago, IL. Abstract SAT-0279

Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells

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Cholesterol is the synthetic precursor of steroid hormones such as estrogens and progestins, which control the growth of many types of human breast cancer. Enzymes responsible for converting cholesterol into steroid hormones are present within breast tumor cells, resulting in local estrogen production. This could lead to tumors becoming resistant to anti-estrogen therapy. In this study we determined whether oxidosqualene cyclase (OSC), an enzyme in the cholesterol biosynthetic pathway, might be targeted to suppress progression of breast cancer cells. Using in silico analysis we previously identified the OSC inhibitor RO 48-8071 (RO), as a potential ligand which could be used to control the progression of estrogen receptor-alpha positive (ERα +ve) breast cancer cells. However, real-time PCR analysis of mRNA from human breast cancer biopsies obtained at various stages of disease did not identify significant differences in OSC expression between tumor tissues or between tumor and normal mammary cells. Nevertheless, since RO reduced tumor cell viability, we examined other potential targets by which it might exert its anti-proliferative effects. Since the growth of hormone-responsive tumors is ERα-dependent, we determined whether RO affected ERα. Using non-human mammalian cells engineered to express human ERα protein and an ERα–responsive luciferase promoter (Indigo Biosciences) we found that RO inhibited 17β-estradiol (E2)-induced ERα responsive luciferase activity. Inhibition was dose-dependent, with an IC_{50} of approximately 10 μM under conditions that were non-toxic to the cells. In order to determine whether RO influenced the biological activity of ERα, we selected treatment conditions (5 μM RO, 16-18h) that did not affect cell viability or influence ERα protein levels. We then treated BT-474 breast cancer cells with 10 nM E2 ± 5 μM RO for 16-18 h and used Western blotting to measure levels of PR, an estrogen responsive gene. RO reduced PR levels in BT-474 cells, confirming that it blocks ERα activity in tumor cells. Real-time PCR and Western blotting revealed no effect of RO on levels of either ERα mRNA or protein. Our findings demonstrate that an important means by which RO suppresses hormone-dependent growth of breast cancer cells is through its ability to arrest the biological activity of ERα. We suggest therefore that our studies support further investigation of RO as a potential therapeutic agent for use against hormone-dependent breast cancers.

Support: Supported by a Dept of Defense Breast Cancer Pgm grant W81XWH-12-1-0191, and by a COR grant from the University of Missouri, Columbia. SMH is the Zalk Missouri Professor of Tumor Angiogenesis.